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LMO4 is highly expressed in breast epithelial cells and is related to cell proliferation and/or invasion in vivo. Because these cellular features are associated with breast carcinogenesis and since LMO4 is overexpressed in more than 50% of breast cancer cases, we hypothesize that LMO4 may play roles in oncogenesis of breast epithelial cells by regulating proliferation, invasion and/or other cellular features. Using LMO4 over-expression or shRNA expression system in vitro, I found that LMO4 play crucial roles in the regulation of cell proliferation and apoptosis of normal mammary gland epithelial cells or breast cancer cells. Furthermore, I have also observed that deletion of LMO4 impaired the function and development of mammary gland in LMO4 conditional knockout mice, indicating that LMO4 protein is necessary for maintaining the normal development of mice mammary gland. In addition, I demonstrated that the LMO4 can modulate TGFβ signaling and regulated the proliferative response of epithelial cells to TGFβ signaling, and thereby linked LMO4 to a conserved signaling pathway that plays important roles in epithelial homeostasis. Under the support of grant, I received excellent training in bioinformatics. By combining previously described functional methods with bioinformatics approaches, we used DNA microarrays to discover LMO4-responsive genes, and identified BMP7 as a key down-stream gene of LMO4. In addition, we also found a significant correlation between LMO4 and BMP7 transcript levels in a large dataset of human breast cancers, providing additional support that BMP7 is a bona fide target gene of LMO4. Finally, we demonstrated that LMO4 binds to HDAC2 and that they are recruited together to the BMP7 promoter. We also suggested a novel mechanism for LMOs; LMO4, Clim2 and HDAC2 are part of a transcriptional complex, and alterations in LMO4 levels can disrupt the complex, leading to decreased HDAC2 recruitment and increased promoter activity. These results strengthen the hypothesis that LMO4 may contribute to the oncogenesis of breast tissue, indicating that our work will play a role in solving the breast cancer problem with the support of the Army Breast Cancer Research Program.

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B. Bibliography of publications and meeting abstracts:

Papers:

1). Ning Wang, Kevin Lin, ***Zhongxian Lu***, Kaye Starr Lam, Xiaoman Xu, Gordon N. Grill and Bogi Andersen. The LIM only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells. ***Oncogene***, 2007, 26(44):6431-41.

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3). Zhengquan Yu, Kevin Lin, Ambica Bhandari, Joel Spencer, Xiaoman Xu, Ning Wang, ***Zhongxian Lu***, Gordon N Gill, Dennis R. Roop, Phillp Wertz and Bogi Andersen. The Grainyhead-like epithelial transactivator Get-1/Grhl3 regulates epidermal terminal differentiation and interacts functionally with LMO4. ***Developmental Biology,*** 2006, 299(1):122-36.

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2). Zhongxian Lu, Kaye Starr Lam, Ning Wang, Xiaoman Xu, Manuel Cortes, and Bogi Andersen. 2005. A Novel Smad-associating Protein, LIM-only protein 4 (LMO4), modulates TGFβ Signaling in Mammary Gland Epithelial Cells. The 2005 Conference of Chao Family Comprehensive Cancer Center, Palm Spring, California, November 11-13.

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INTRODUCTION

My training program contains two distinct components: molecular research in breast cancer and computational biology and bioinformatics. The two components will be integrated because analyses of data obtained from my laboratory research will be one of my entry points into computational biology. In addition, results from the computational part of my project will spawn biological experiments. **A**. My training in computational biology will involve formal course work from the UCI Bioinformatics Training Program. These courses include *Basic Statistics (Math 7, 4u), Introduction to Computer Science (ICS 21, 6u), Representations and Algorithms for Molecular Biology (ICS 277A, 4u)* and *Probabilistic Modeling of Biological Data (ICS 277B, 4u)***. B.** My molecular laboratory research training focuses on the LIM-only factor (LMO) 4 genes. LMO4 belongs to a family of four mammalian LMO proteins, which are only composed of two LIM domains[1]. LMOs are thought to act as adapter molecules in transcriptional complexes, tethering the co-activators CLIM (Nli/Ldb) to various DNA-binding proteins[2]. LMOs family proteins show a crucial role not only during development, but also in tumorigenesis. LMO1 and LMO2 act as oncogenes in acute lymphoblastic leukemia[3]. LMO4 is also referred to as Human Breast Tumor Autoantigen based on that LMO4 was also first isolated from breast cancer tissue and overexpressed in more than 50% of breast cancer cases[4, 5]. Furthermore, LMO4 interacts with BRCA1 and inhibits the activation of BRCA1[6]. In study comparing expression profiles in estrogen positive and negative breast cancer, LMO4 was found in a panel of genes that strongly predicted estrogen negative status of breast cancer. My hypothesis is that, analogous to the role of LMO2 in leukemia, LMO4 overexpression promotes oncogenesis of breast epithelial cells by deregulating one or more of the following cellular features: differentiation, proliferation, apoptosis or invasion. In addition, we hypothesize that LMO4 acts, at least in part, by interacting with BRCA1, thereby interfering with the regulation of BRCA1 target genes.

Our specific aims were: #1. To test the effects of LMO4 overexpression or LMO4 interference in breast cancer by conditional expression systems and an interfering RNA plasmid system to increase and decrease, respectively, LMO4 protein levels in breast cancer cell lines. #2. To use gene expression profiling in MCF-7 breast cancer cells to elucidate the mechanisms of action for LMO4 overexpression. To gain insights into how LMO4 acts at a molecular level, we will use Affymetrix microarrays to define the profile of genes altered by LMO4 in breast cancer cells.

BODY

Task 1. **Test the phenotypic effects of conditional LMO4 overexpression and LMO4 interference in human breast cancer cells.** Tasks 1.1 to 1.3 are technical in nature and are described in previous progress report and our papers[7-9], which are included with the Appendix. Task 1.4 was also described in our papers (7).

1.1 Created breast cancer cell lines overexpressing LMO4.

The initial plan was to create conditional overexpression cell lines: MCF7-LMO4-TetOff. In addition, I have created a retroviral gene transduction system which has high

efficiency of overexpressing LMO4 in breast cancer cells and primary epithelial cells. These LMO4 overexpression cell lines were used efficaciously in my experiments.

1.2 Established breast cancer cell lines that express LMO4-RNAi.

Using the construct of LMO4 shRNA, I have established permanent cell lines of MD-MBA-231 and T47D, which stably expressed LMO4-RNAi. In addition, I also employed a retroviral gene transduction system to express an Engailed-LMO4 fusion protein to block LMO4 regulation in breast cancer cells or primary epithelial cells. The fusion of the engrailed repression domain to LMO4 creates a strong dominant-negative molecule, predicted to actively repress LMO4 target genes.

1.3 Tested the phenotypic effects of increased or decreased LMO4 protein expression in normal mammary gland epithelial cells and breast cancer cells.

The initial plan was to test only the biological effect of LMO4 in breast cancer cell lines. However, the roles of LMO4 in normal mammary gland were unclear. Thus, I have also studied the phenotypic effects of LMO4 in human primary breast epithelial cells using retroviral gene transduction system. In addition, I have also investigated the co-effects of LMO4 and TGFβ on human primary breast epithelial cells based on our finding that LMO4 binds to Smad protein and modulates TGFβ signal.

1.4 Test the phenotypic effects of conditional deletion of LMO4 in mouse mammary gland.

The original project didn't involve testing the phenotypic effects of conditional deletion of LMO4 in mouse mammary gland. But, we found that LMO4 plays important roles in cell proliferation and apoptosis in mammary gland normal epithelial cells and cancer cells. Thus, to full understand the physiological roles of LMO4 in mammary gland, we have created and characterized two lines of transgenic mice: Wap-Cre-*LMO4*$^{fl/fl}$ and MMTV-Cre-*LMO4*$^{fl/fl}$.

LMO4 knockout mice die during embryogenesis or at birth, precluding their use for studying the role of LMO4 in postnatal mammary gland development[9]. So, we employed Whey Acidic Protein (WAP)-Cre[10] or Mouse Mammary Tumor Virus (MMTV)-Cre[10] to conditionally delete the *LMO4* gene in mammary glands of mice. Although both the WAP and the MMTV promoters are active in mammary gland epithelial cells, these two promoters have distinct features. The MMTV-Cre transgene is expressed in all major epithelial subtypes (luminal and myoepithelial cells), while expression of the WAP-Cre transgene is limited to the secretory epithelium . Also, the MMTV-Cre transgene is active at a low constitutive level during ductular growth in virgin mice, whereas the WAP promoter is only active during midpregnacy and later; the WAP promoter is not active in the mammary gland of virgin mice . Therefore, we investigated mammary gland development of mice with MMTV-Cre-mediated deletion of *LMO4* (MMTV-Cre-*LMO4*$^{fl/fl}$) during virgin development, as well as in pregnancy and lactation of the first pregnancy. Mice with WAP-Cre-mediated deletion of *LMO4* (Wap-Cre-*LMO4*$^{fl/fl}$) were studied during pregnancy and lactation of the second pregnancy.

We interbred floxed *LMO4* mice[11] with WAP-Cre or MMTV-Cre transgenic mice to achieve two types of Cre recombinase-mediated deletion of the *LMO4* gene in mammary glands of mice. Then, total RNA was isolated from the inguinal mammary glands of knockout and heterozygous control mice. By means of quantitative real-time PCR, we found that LMO4 mRNA levels were dramatically decreased in both MMTV-Cre-*LMO4*$^{fl/fl}$ and Wap-Cre-*LMO4*$^{fl/fl}$ mice (Fig. 1A and B), suggesting that LMO4 levels are effectively lowered in both knockout models.

To test whether deletion of the *LMO4* gene interfered with the function of the mammary gland, we studied the growth of pups nursed by MMTV-Cre-*LMO4*$^{fl/fl}$ female mice, and compared to pups nursed by control females (MMTV-Cre-*LMO4*$^{fl/+}$). Pups nursed by *LMO4* knockout mothers show significantly decreased weight gain (Fig. 1C). The quantitative real-time PCR showed that LMO4 knockout mice have low mRNA expression level of milk protein (whey and casein) compared to their control (data not shown). These results indicated deletion of the *LMO4* gene impaire the function of the mammary gland. Whole mount and histological analyses of Wap-Cre-*LMO4*$^{fl/fl}$ mammary glands showed impaired lobuloalveolar development at days 13.5 and 17.5 of pregnancy, and at the first day of lactation (Fig. 2A). A similar phenotype was observed in the mammary glands of MMTV-Cre-*LMO4*$^{fl/fl}$ mice (Fig. 2B), and in this model, striking impairment of lobuloalveolar development was observed as early as day 5.5 of pregnancy (Fig. 2B; top panels). A decrease in ductular growth was also observed in 3-week old MMTV-Cre-*LMO4*$^{fl/fl}$ virgin mice (Fig. 3A). The similar pregnancy phenotypes of both types of *LMO4* knockout mice provide strong support for an important role of *LMO4* in lobuloalveolar development of the mammary gland. These data, in combination with previous studies[12], demonstrate that the *LMO4* gene is important for lobuloalveolar development during pregnancy, and that deletion of the *LMO4* gene results in decreased lobuloalveolar structures.

To determine the cause of decreased lobuloalveolar development in *LMO4* knockout mice, we investigated proliferation and apoptosis of mammary epithelial cells with Ki67 immunostaining and terminal deoxynucleotidyltransferase-mediated dUTP end-labeling (TUNEL) assays, respectively (Fig.4). In the normal mammary gland, expression of the proliferation marker Ki67 peaks in mid-pregnancy and by lactation day 1 there are few positive cells. Mammary epithelial cell proliferation was reduced by about 50% at pregnancy days 13.5 and 17.5 in both MMTV-Cre-*LMO4*$^{fl/fl}$ and Wap-Cre-*LMO4*$^{fl/fl}$ mice (Fig. 4A, B). In Wap-Cre-*LMO4*$^{fl/fl}$, apoptosis of mammary epithelial cells was significantly increased in mid-pregnancy at day 13.5, and a similar, but non-significant trend was also observed in late pregnancy at day 17.5 (Fig.5). Consistent with this finding, we noticed large spaces with loss of epithelial structures in about half of Wap-Cre-*LMO4*$^{fl/fl}$ mice at lactation day 1 (Fig.3B). Increased apoptosis was not observed in MMTV-Cre-*LMO4*$^{fl/fl}$ mammary glands (data not shown), perhaps related to the different kinetics of deletion in the two types of *LMO4* knockout mice.

To gain insights into the molecular event underlying the cellular phenotype of *LMO4* knockout mice, we investigated the expression of cell cycle regulators known to be important for mammary gland development. Increased expression of p15 was found in the mammary glands of *LMO4* knockout mice both during mid-pregnancy and at day one

of lactation (Fig.6). Since p15 is a cell cycle inhibitor, increased p15 expression may lead to decreased proliferation and increased apoptosis in *LMO4* knockout mice. These effects are specific because we found no alterations in the expression of p21, p27, cyclin D and Myc (data not shown) –all cell cycle regulators implicated in mammary gland development .

Together, our results indicate that the *LMO4* gene plays a role in maintaining proliferation and survival of mammary epithelial cells during lobuloalveolar development. Decreased cellular proliferation during early and mid-pregnancy appears to be the main mechanism underlying decreased lobuloalveolar development in *LMO4* knockout mice, but increased apoptosis also contributes to the phenotype.

Task 2. **To use gene expression profiling in MCF-7 breast cancer cells to elucidate the mechanisms of action for LMO4 overexpression.** Tasks 2.1 to 2.3 are technical in nature and are described in previous progress report and our papers (7-8), which are included with the Appendix.

2.1 Discovered LMO4 responsive genes in MCF-7 cells overexpressing LMO4.

The initial plan was to discover LMO4 responsive genes in MCF-7 cell overexpressed LMO4. Since LMO4 action is based on its interactions with Clims in epidermal cell, we reasoned that interfering with Clim function in MCF-7 cells would help identify *bone fide* LMO4-responsive genes. We therefore created MCF7-DN-Clim-TetOff cell lines where a dominant-negative (DN) Clim protein is expressed. Then, we identified the genes responding to LMO4 and DN-clim.

2.2 Defined BMP7 is a key target gene of LMO4.

This was accomplished early in the project, see previous progress report and (7)

2.3 Elucidated the mechanisms of action for LMO4 overexpression.

This was accomplished early in the project, see previous progress report and (7)

2.4 Studied the functional relationship between LMO4 and BRCA1.

The initial plan was to discover BRCA1 responsive gene in breast cancer cells (such as MDA-MB-231) which was deleted BRCA1 protein by siRNA. But, just at beginning of my projects, Bae et al reported the gene profiling in breast cancer cells stably expressing BRCA1 siRNA[13, 14]. Therefore, we combined their databases with our database and analyzed the overlapping of responsive genes. However, we found there are too few common responsive genes of LMO4 and Brca1 to provide any useful information (data not shown). LMO4 regulates cell proliferation and apoptosis of both normal epithelial cells and cancer cells of breast, indicating that normal development and cancer may share conserved mechanisms.

However, no systematic approach has been applied to analyze the extent and global characteristics of the overlap in gene expression between developing tissues and cancer. So, taking advantage of my bioinformatics training, I propose to develop novel algorithms capable of processing information from mammary gland development and breast cancer gene profiling studies, as well as several other sources, to create a ranked

list of genes that are candidates for breast cancer genes **(see the following describing in 2.5)**

5. Analyzed the overlapping gene between mammary gland development and tumorigenesis.

Our studied demonstrated that LMO4 plays crucial roles in both normal development and tumorigenesis of mammary gland. Because both processes, development and neoplasia, involve alterations in cell proliferation, cell differentiation, neovascularization, cell migration and invasion, as well as cell apoptosis, it is not surprising that LMO4 regulates cell proliferation and apoptosis of both normal epithelial cells and cancer cells of breast. Recently, it has become increasingly evident that normal development and cancer may share conserved mechanisms. However, it still remains unknown to which extent global characteristics of gene expression overlap between developing tissues and cancer.

Bioinformatics exhibit excellent power in analysis of global and systematic gene expression profiling in tumorigenesis and has been applied in clinical prognosis, mining of novel cancer genes, etc. So, based on my bioinformatics training, I will be developing a new approach to aid in the discovery of novel cancer modulated genes by analyzing the relationship of expression profiles between mammary gland development and tumorigenesis.

Now, I have refined a mammary gland associated gene datasets (Fig 7) based on a published microarray database[15]. The data was filtered by P&M to exclude the "Absent" probes (Fig 7A). 6951 probes were identified and called "Present" probes. Then, we employed the following two methods to assess different cutoffs for identifying a gene as mammary gland-development-associated: a literature-based validation and *a statistical analysis of* genes whose expression levels were significantly different between at least two time points. For literature-based validation, we compiled a list of genes whose expression patterns have been shown to be mammary gland- development-dependent (data not show). We found that >90% of these genes have $P < 0.01$ (ANOVA across all time points). However, p value less than 0.01 also cover more than 85% of Present probes, probably because there are too many time points. So, we used another criterion: the ratio of maxium expression to minimum expression of genes. We found that >75% of these genes have max/min >3, and biggest Odd ratio of literature-based mammary gland-cycle associated genes (Fig 7B). This analysis yielded a "refined" data set of 2939 probes that varied significantly across the time course, hereafter termed mammary gland developmental cycle associated genes.

I also collected the "altered" genes whose expression is altered in breast tumor compare to normal mammary gland or reference pool from published microarray datasets[16-20]. Unigene ID was used to establish correspondence between anthologies in mouse and human. To evaluate the performance of our model**,** we created a preliminary reference list of known cancer genes from published sources that have cataloged cancer genes, the Atlas of Genetic and Cytogenetics in Oncology and

Haemotology(www.infobiogen.fr/services/chromcancer/index.htm), the Cancer GeneticsWeb (www.cancerindex.org/geneweb), and the Tumor Gene Database (condor.bcm.tmc.edu/oncogene.html).

Using my refined mammary gland associated genes and the "altered" genes in breast tumors; I have done some preliminary experiments. I found that known cancer genes are enriched in the group of genes showing developmental expression compared to those showing (Fig.7C). In addition, developmental genes whose expression is consistently altered in multiple databases have a higher probability of being cancer genes (Fig. 8). Of all developmental genes showing altered expression in all five tumor datasets, approximately 60% are known cancer genes. In contrast, of developmental genes with altered expression in only one tumor datasets, 20% are known cancer genes (Fig. 8, blue bars). However, for non-development genes, the percentage of known cancer genes shows no significant increase (Fig.8, red bars). This result indicates that the developmental regulation of a given gene increases the probability that it is involved in cancer, which is consistent with other studies. These studies will open new research projects and provide some preliminary data for advanced grant application.

Task 3. **Formal training in bioinformatics.**

During the past three year, I completed the following course: *Basic Statistic*s, *Representations and Algorithms for Molecular Biology*, *Introduction to Computer Science* and *Probabilistic Modeling of Biological Data*. Based Java language, fundamental concepts related to computer software design and construction were learned, and skills to design programs were developed. These courses greatly improve my ability in working on computational experiments (such as understanding and designing program to analyze gene expression profiling in microarray database, which is the major experiment in specific aim #2.). I have also obtained significant practical experience in bioinformatics as my progress report indicates. I statistically evaluated a large microarray dataset and used this analysis to discover LMO4 target genes, and demonstrated the validity of BMP7 as an LMO4 target gene. In addition, I used computational methods to study the correlation between LMO4 transcript levels and expression of other genes in a large breast cancer dataset. Furthermore, using bioinformatics approaches, I will analyze the relationship of gene expression profile between mammary gland development and tumorigenesis, and build a powerful model to find novel cancer modulated genes based on the intrinsic molecular relation between development and tumorigenesis of mammary gland.

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that LMO4 plays a crucial role in cell survival of mammary gland cells or breast cancer cells by regulating cell apoptosis.
2. Demonstrated that LMO4 protein is necessary for maintaining the mammary gland development, based on the observation of impaired development of mammary gland in LMO4 conditional knockout mice.
3. Defining the regulation mechanisms of LMO4 on cell growth, specifically that LMO4 regulated cell growth by modulating TGFβ signal.
4. Demonstrated that LMO4 modulates TGFβ signaling pathway by interaction with Smad proteins.
5. Defined BMP-7 as a key LMO4 target gene that can mediate some of the effects of LMO4 on breast cancer cells.
6. Discovered novel regulatory mechanisms for LMO4 gene regulation, involving histone deacetylases, in mammary gland development and breast cancer progression.

REPORTABLE OUTCOMES

1. Permanent breast cancer cell lines of LMO4 overexpression.
2. Permanent breast cancer cell lines of LMO4 siRNA.
3. A dominant-negative LMO4 construct: Engrail fused to LMO4.
4. The retrovirus infection system of Engrail-LMO4 to control genes.
5. Gene expression database in MCF-7 which over-expressed LMO4.
6. Transgenic mouse model of conditional deletion of the LMO4 gene in mammary gland with WAP-cre.
7. Transgenic mouse model of conditional deletion of the LMO4 gene in mammary gland with MMTV-cre.
8. Manuscripts and Abstracts:

Manuscripts:

1). Ning Wang, Kervin Lin, ***Zhongxian Lu***, Kaye Starr Lam, Xiaoman Xu, Gordon N. Grill and Bogi Andersen. The LIM only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells. ***Oncogene***, 2007, 26(44):6431-41.

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CONCLUSION

In summary, with the support of the Army Fellowship Award, I have obtained excellent training in both molecular research in breast cancer and computational biology; I am acquiring expertise in working on the breast cancer problem at a high level. In the past three years, I made significant progress on specific aims of my proposal, and my training in breast cancer has been greatly enhanced. I have published one first-author paper and two co-author papers, and several abstracts, which describe my findings involving LMO4 roles in breast cancer. **My major achievements are described as following: (1)** I have demonstrated that LMO4 regulates cell proliferation and apoptosis dependent on the type of breast cancer cells in vitro, using gene over-expression or shRNA expression system. **(2)** I have found LMO4 plays crucial roles in cell proliferation and apoptosis of mammary gland normal epithelial cell. **(3)** I have also observed that deletion of LMO4 impaired the function and development of mammary gland in vivo, indicating LMO4 protein is necessary for maintaining the normal development of mice mammary gland. **(4)** I have completed the screen for LMO4-regulated genes, using microarray technology in MCF-7 cells overexpressing LMO4, and defined several LMO4

direct target genes. **(5)** I have defined that BMP7 is a key down-stream gene of LMO4, and contributes to LMO4-induced apoptosis. **(6)** I found that LMO4 modulates TGFβ signaling as a part of Smad-DNA complex and promotes TGFβ inhibition of cell proliferation of mammary gland epithelial cell, suggesting LMO4 may contribute to the oncogenesis of breast tissue. **(7)** I have discovered that LMO4 modulates the recruitment of HDAC2 to the BMP7 promoter, suggesting a novel mechanism for LMO-mediated stimulation of gene expression. LMO4 is a part of a transcription complex containing LMO4, Clim2 and HDAC2, which is sensitive to stoichiometry of components such that either overexpressing or lowering of LMO4 leads to decreased recruitment of HDAC2 and increased promoter activity.

In conclusion, we demonstrated a crucial role for LMO4 in cell proliferation and apoptosis in mammary gland development and tumorigenesis, and discovered the mechanism of LMO4 regulation using molecular biology combined with bioinformatics approaches. These results strengthen the hypothesis that LMO4 may contribute to the oncogenesis of breast tissue, and indicate that this work will play a role in solving the breast cancer problem with the support of the Army.

Furthermore, LMO4 regulates cell proliferation and apoptosis of both normal epithelial cells and cancer cells of breast, indicating that normal development and cancer may share conserved mechanisms. However, no systematic approach has been applied to analyze the extent and global characteristics of the overlap in gene expression between developing tissues and cancer. So, based on my bioinformatics training, I propose to develop novel algorithms capable of processing information from mammary gland development and breast cancer gene profiling studies, as well as several other sources, to create a ranked list of genes that are candidates for breast cancer genes. These studies will open new research projects and provide preliminary data for advanced grant application.

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Fig. 1

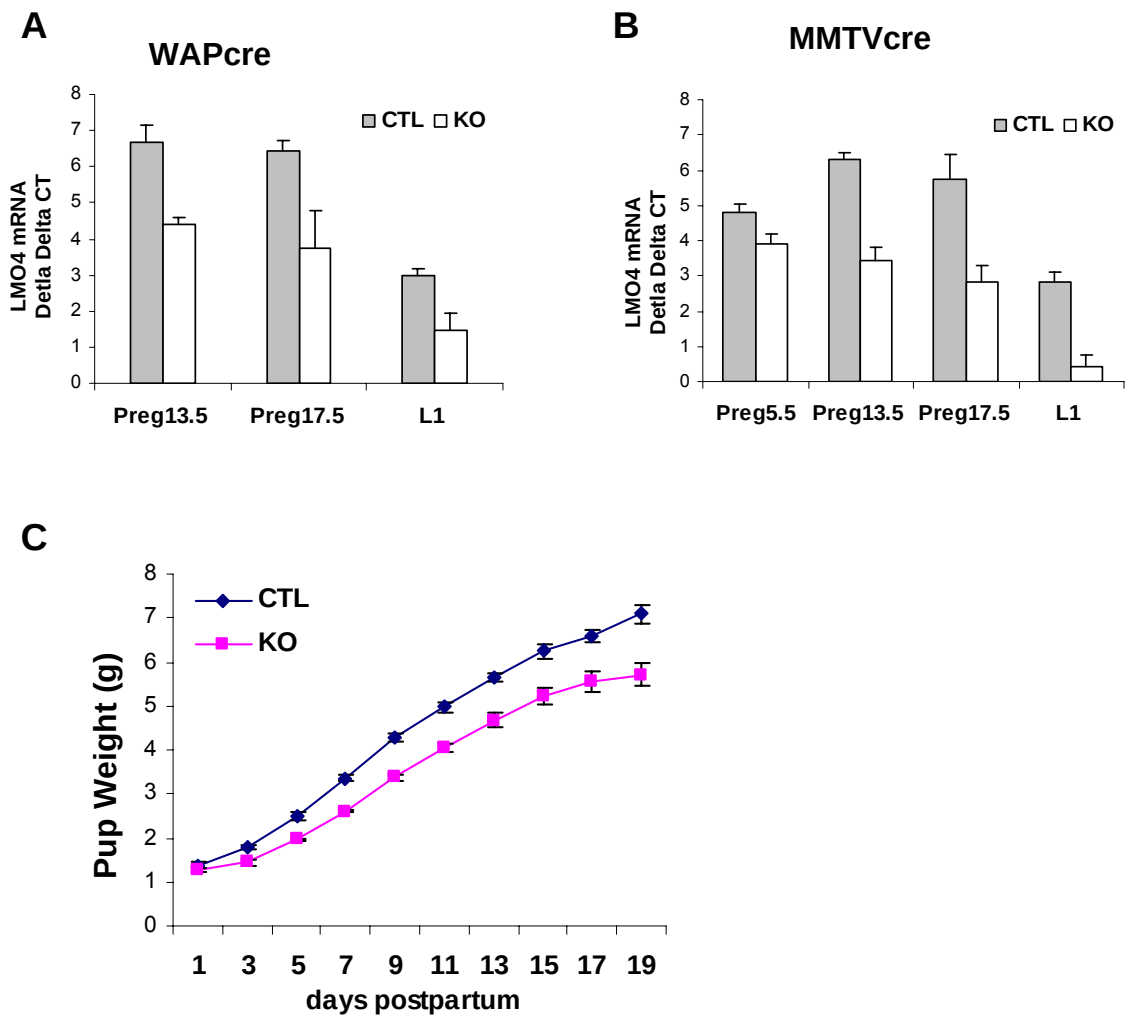


Fig. 1 Deletion of the LMO4 gene in mammary glands of mice impairs lactation function (**A**) LMO4 mRNA levels relative to 18S rRNA in Wap-Cre-*LMO4fl/+* (control) and Wap-Cre-*LMO4fl/fl* (KO) mice from indicated stages of mammary gland development. (**B**) LMO4 mRNA levels relative to 18S rRNA in MMTV-Cre-*LMO4fl/+* (control) and MMTV-Cre-*LMO4fl/fl* (KO) mice from the indicated stages of mammary gland development. Transcripts in A and B were measured with quantitative real-time PCR from at least three samples for each condition. (**C**) Growth curves of offsprings of MMTV-Cre-*LMO4fl/+* (control) and MMTV-Cre-*LMO4fl/fl* (KO) female mice during the first 19 days of lactation. Thirty mice from four control litters and 23 mice from three knockout litters were weighed every other day. There was significant difference ($P<0.01$) in weight at each time point except day 1. Data in panels A, B and C represents mean and SEM. KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control.

Fig. 2

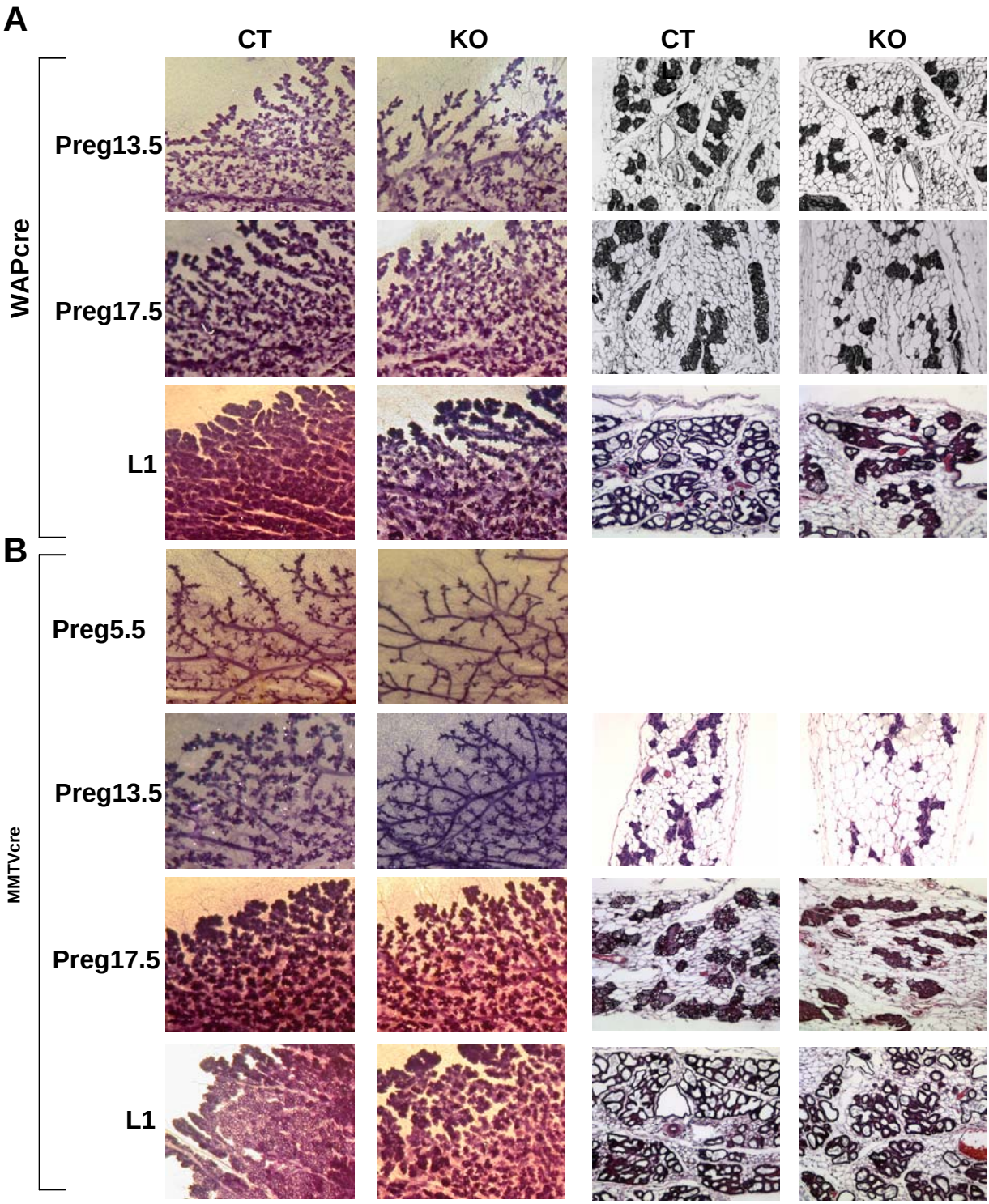

A
CT
KO
CT
KO
WAPcre
Preg13.5
Preg17.5
L1
B
MMTVcre
Preg5.5
Preg13.5
Preg17.5
L1

FIG. 2 Deletion of the LMO4 gene in mammary glands of mice leads to impaired lobuloalveolar development. Whole mount (first two colums) and histological analyses (last two columns, 100X magnification) of the fourth inguinal mammary gland from MMTV-Cre-*LMO4fl/fl* **(A)** and Wap-Cre-*LMO4fl/fl* **(B)** mice and their controls (the genotypes of controls for MMTV-Cre-*LMO4fl/fl* and Wap-Cre-*LMO4fl/fl* are MMTV-Cre-*LMO4fl/+,* and Wap-Cre-*LMO4fl/+*, respectively). KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control.

Fig. 3

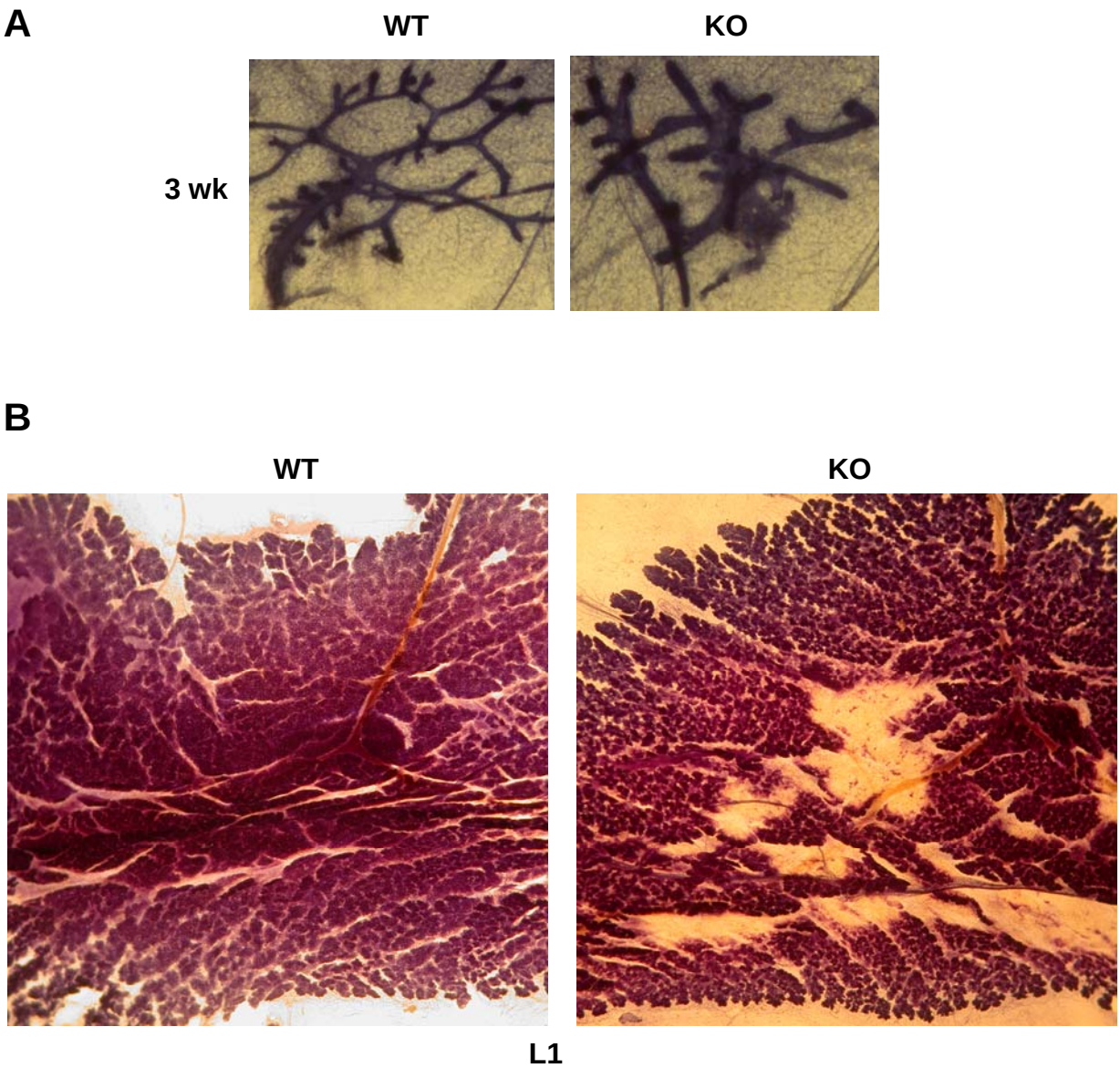
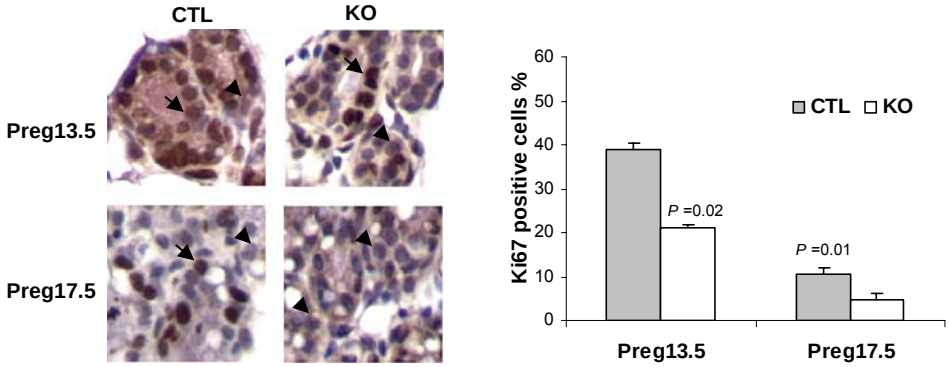


Fig. 3 Conditional deletion of the LMO4 gene leads to impaired structure of mammary glands in mice. **(A)**Three-week-old MMTV-*Cre-LMO4fl/fl* mice show decreased ductal development and decreased number of end buds compared to their controls (MMTV-*Cre-LMO4fl/+*). **(B).** There are large spaces with loss of epithelial structures in about half of Wap-Cre-*LMO4fl/fl* mice at lactation day 1. Whole mount analysis. KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control.

Fig. 4

A. Cell proliferation decreased in WAPcre knockout LMO4 mice (KI67 staining)



B. Cell proliferation decreased in MMTVcre knockout LMO4 mice (Ki67 staining)

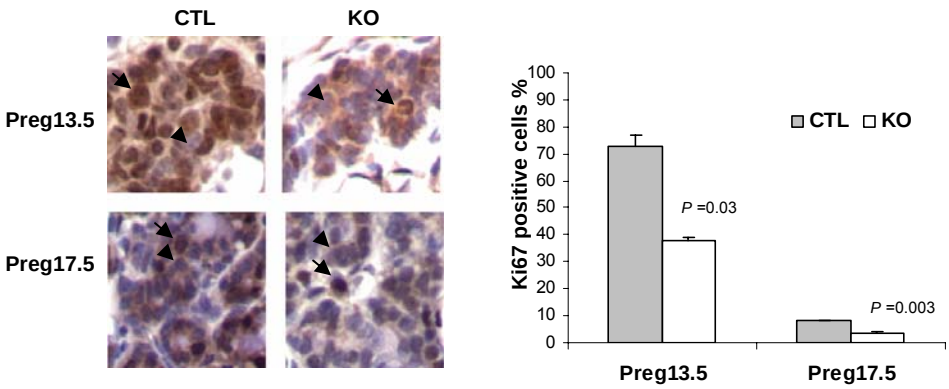


FIG. 4 Decreased lobuloalveolar development in LMO4 knockout mice is due to decreased proliferation of mammary epithelial cells.

(**A**) Ki67 antibody staining of paraffin-embedded sections of mammary glands from control and Wap-Cre-*LMO4fl/fl* mice from the indicated stages. Right diagram show quantification of Ki67 positive cells in control and Wap-Cre-*LMO4fl/fl* mice from the indicated stages. (**B**) Ki67 antibody staining of paraffin-embedded sections of mammary glands from MMTV-Cre-*LMO4fl/fl* and their control mice from the indicated stages. Right diagram quantification of Ki67 positive cells in MMTV-Cre-*LMO4fl/fl* mice and their control mice from the indicated stages. For the quantification in B and D, 500 cells were counted in at least 3 wild type and knockout mice. Arrows and arrowheads in A and B point to exemplary positive and negative cells, respectively. KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control.

Fig. 5

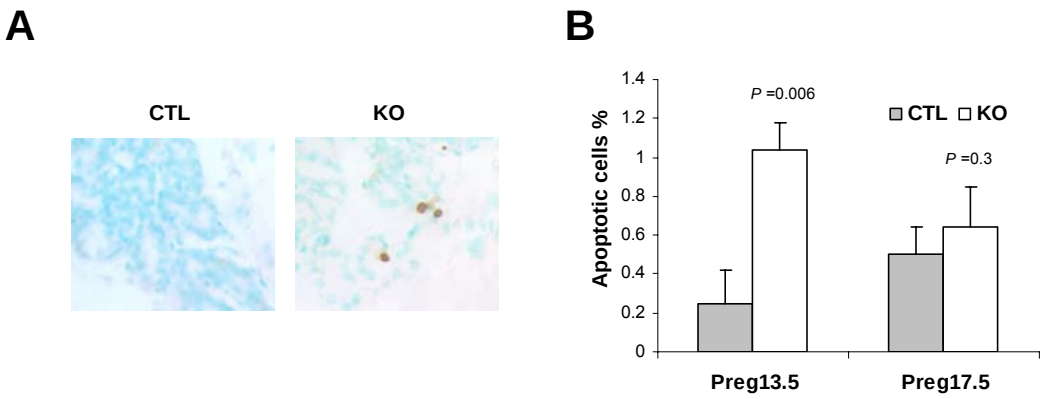


Fig. 5 Cell apoptosis increased in WAPcre knockout LMO4 mice. (**A**) TUNEL staining of paraffin-embedded sections of mammary glands from Wap-Cre-*LMO4fl/fl* mice and their control mice from the indicated stage. (**B**) Quantification of TUNEL positive cells in Wap-Cre-*LMO4fl/fl* mice and their control mice from the indicated stages. For these studies, 2000 cells were counted in at least 4 wild type and knockout mice. KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control.

Fig. 6

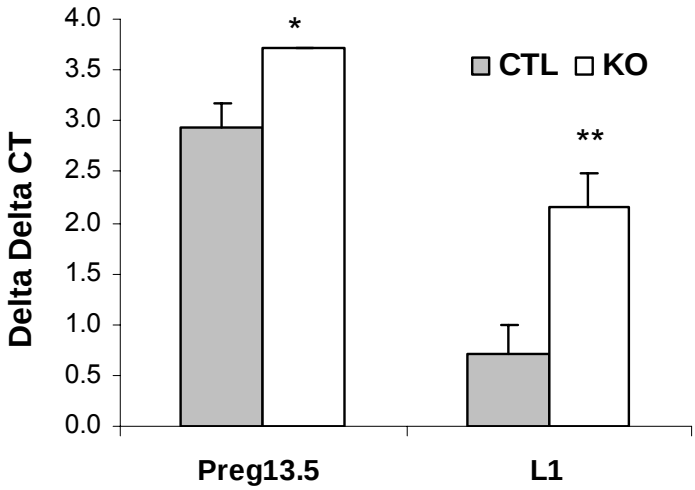


Fig. 6 Deletion of LMO4 increased P15 protein expression. p15 mRNA levels normalized to 18S rRNA expression were quantified with real-time PCR in MMTV-Cre-LMO4fl/fl mice. The results are from at least three mice for each condition. The data in all panels represents mean and SEM; *P*-values were calculated using t-test.(*<0.05; **<0.01), KO, knockout; L, lactation; Preg, Pregnancy; CTL, Control..

Fig. 7

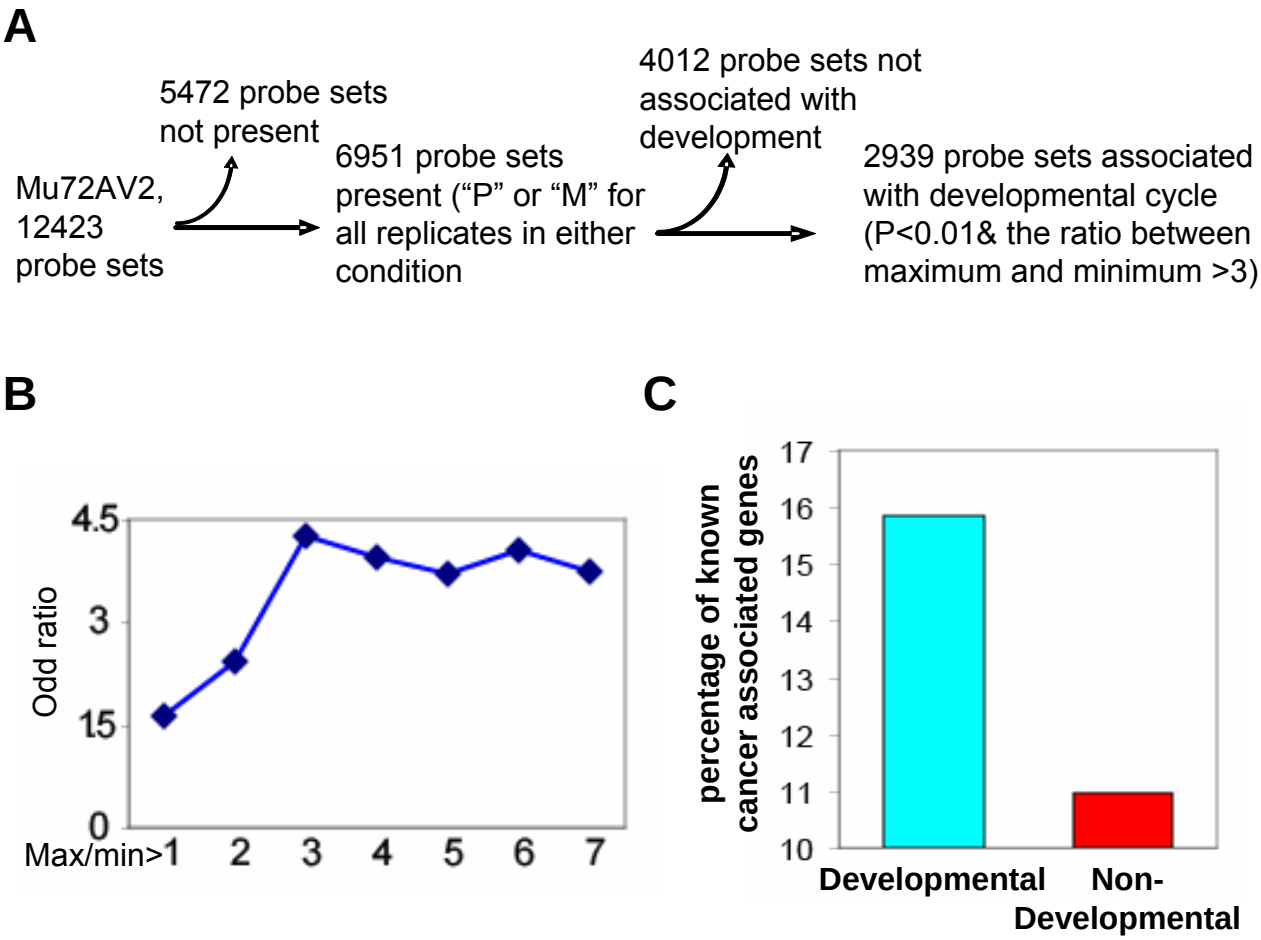


Fig. 7 determination of mammary gland developmental associated genes. **A**. Overview of data processing for refined developmental gene set. **B.** Refined developmental associated gene included 73% of known developmental genes compiled based on literature. **C**. Development associated genes contain significantly higher number of known cancer associated genes.

Fig. 8

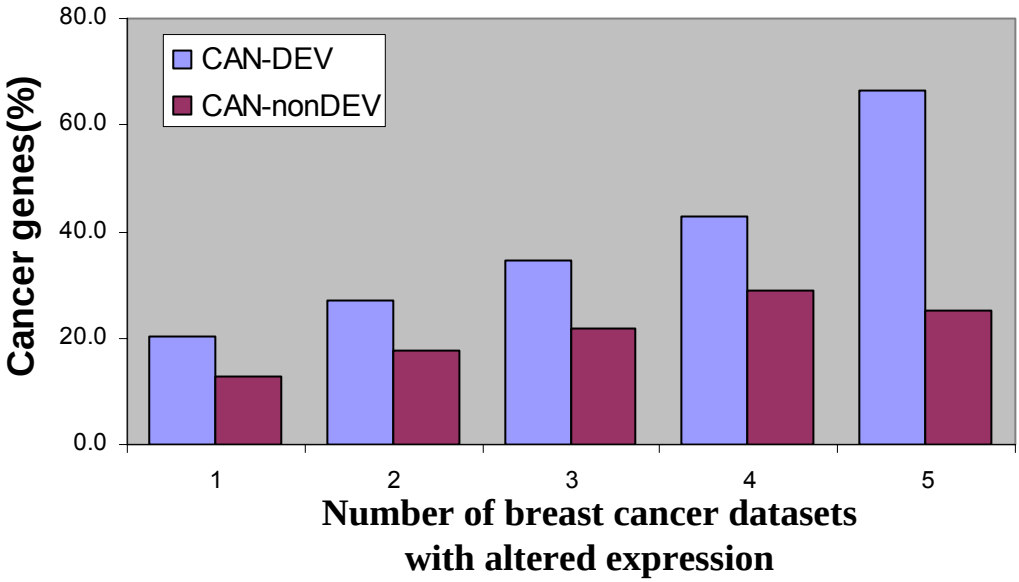


Fig.8 The relationship between known cancer genes and developmental regulation as a function of consistencey in alteration among five different breast cancer databases. The X-axis shows the number of breast cancer microarray datasets (out of 5) with altered gene expression. The Y axis shows the proportion (in %) of known cancer genes in each group. Genes were classified as either developmentally regulated (see text; blue bars) or not regulated during development (see text; red bars).

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ORIGINAL ARTICLE

The LIM-only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells

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The nuclear LIM-only protein 4 (LMO4) is upregulated in breast cancer, especially estrogen receptor-negative tumors, and its overexpression in mice leads to hyperplasia and tumor formation. Here, we show that deletion of *LMO4* in the mammary glands of mice leads to impaired lobuloalveolar development due to decreased epithelial cell proliferation. With the goal of discovering potential LMO4-target genes, we also developed a conditional expression system in MCF-7 cells for both LMO4 and a dominant negative (DN) form of its co-regulator, cofactor of LIM domains (Clim/Ldb/Nli). We then used DNA microarrays to identify genes responsive to LMO4 and DN-Clim upregulation. One of the genes common to both data sets was bone morphogenic protein 7 (*BMP7*), whose expression is also significantly correlated with LMO4 transcript levels in a large dataset of human breast cancers, suggesting that *BMP7* is a *bona fide* target gene of LMO4 in breast cancer. Inhibition of BMP7 partially blocks the effects of LMO4 on apoptosis, indicating that BMP7 mediates at least some functions of LMO4. Gene transfer studies show that LMO4 regulates the BMP7 promoter, and chromatin immunoprecipitation studies show that LMO4 and its cofactor Clim2 are recruited to the BMP7 promoter. Furthermore, we demonstrate that HDAC2 recruitment to the BMP7 promoter is inhibited by upregulation of LMO4 and that HDAC2 knockdown upregulates the promoter. These studies suggest a novel mechanism of action for LMO4: LMO4, Clim2 and HDAC2 are part of a transcriptional complex, and increased LMO4 levels can disrupt the complex, leading to decreased HDAC2 recruitment and increased promoter activity.
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Keywords: breast cancer; lim domain; LMO; proliferation; apoptosis; HDAC

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Introduction

Lim-only protein 4 (LMO4) belongs to a family of four mammalian nuclear LMOs characterized by the presence of two tandem LIM domains and no other functional domains (Bach, 2000). LIM domains mediate protein–protein interactions, and all nuclear LMOs bind with high affinity to a transcriptional cofactor of LIM domains (Clim)/LIM domain binding protein (Ldb)/Nuclear LIM interactors (Nli), which enhance transactivation by LIM homeodomain factors and some other DNA-binding proteins (Bach, 2000). LMOs are proposed to function as transcriptional activators by recruiting Clims to DNA-binding proteins. LMOs can also feedback inhibit LIM homeodomain function by displacing Clim from LIM homeodomain proteins (Milan *et al*., 1998). Recently, chromatin-modifying transcriptional cofactors, including HDACs have been identified as LMO4 interactors (Singh *et al*., 2005).

LMOs play critical roles in distinct pathways of mammalian development and deregulation of their expression is linked to oncogenesis (Rabbitts *et al*., 1999). *LMO4* has been implicated in the cause or progression of breast cancers (Sum *et al*., 2005b), squamous cell carcinomas of oral cavity (Mizunuma *et al*., 2003) and primary prostate cancers (Mousses *et al*., 2002). The *LMO4* gene is most highly expressed in mammary epithelial cells during midpregnancy (Wang *et al*., 2004), a stage of active proliferation and invasion; interference with the protein (Wang *et al*., 2004) or deletion of the gene (Sum *et al*., 2005c) leads to impaired lobuloalveolar development of the mammary gland. LMO4 is overexpressed in over half of primary breast cancers and its expression is associated with a worse prognosis (Visvader *et al*., 2001; Sum *et al*., 2005b). In addition, overexpression of LMO4 in the mammary gland of mice leads to hyperplasia and intraepithelial neoplasia (Sum *et al*., 2005b). Furthermore, the *LMO4* gene is activated by heregulin (Wang *et al*., 2004) and is overexpressed in Her2-mediated tumors (Landis *et al*., 2005). Together, these observations demonstrate that LMO4 has critical functions in mammary epithelial cells.

While recent studies indicate that LMO4 promotes both normal development and tumor formation in the

mammary gland, the molecular mechanisms underlying these effects remain unknown. In the present study, we identify bone morphogenic protein 7 (*BMP7*) as a target gene of LMO4 in breast cancer cells. We show that LMO4 associates with the *BMP7* promoter and that alterations in the levels of LMO4 can regulate the recruitment of histone deacetylase HDAC2 to this promoter. Our studies have identified a novel transcriptional mechanism for LMO4 in which it activates transcription by decreasing recruitment of HDAC2 to the *BMP7* promoter.

Results

Deletion of LMO4 *causes decreased proliferation of mammary epithelial cells and impaired lobuloalveolar development*

LMO4 knockout mice die during embryogenesis or at birth (Hahm *et al*., 2004; Lee *et al*., 2005; Sum *et al*., 2005c), precluding their use for studying the role of *LMO4* in postnatal mammary gland development. We therefore interbred floxed *LMO4* mice (Lee *et al*., 2005) with whey acidic protein (WAP)-Cre (Wagner *et al*., 1997) and mouse mammary tumor virus (MMTV)-Cre (Wagner *et al*., 1997) transgenic mice to achieve two types of Cre recombinase-mediated deletion of the *LMO4* gene within mammary glands of mice (Supplementary Figure S1). Whole-mount and histological analyses were used to study the effect of *LMO4* deletion on mammary gland development. Lobuloalveolar structures of WAP-*Cre-LMO4*$^{fl/fl}$ mammary glands were decreased at days 13.5 and 17.5 of pregnancy, and at the first day of lactation (Figure 1a). MMTV-*Cre-LMO4*$^{fl/fl}$ mice showed striking impairment of lobuloalveolar development as early as day 5.5 of pregnancy (Figure 1b, top panels). A decrease in ductular growth was also observed in 3-week-old MMTV-*Cre-LMO4*$^{fl/fl}$ virgin mice (Supplementary Figure S2). Consistent with impaired lobuloalveolar development, pups nursed by *LMO4* knockout mothers show significantly decreased weight gain (Supplementary Figure S3). Together, these studies indicate that *LMO4* is a modulator of mammary gland development.

To determine the cause of decreased lobuloalveolar development in *LMO4* knockout mice, we investigated proliferation of mammary epithelial cells with Ki67 immunostaining. In the normal mammary gland, expression of the proliferation marker Ki67 peaks in midpregnancy and by lactation day 1 there are few positive cells. Mammary epithelial cell proliferation was reduced by about 50% at pregnancy days 13.5 and 17.5 in both MMTV-*Cre-LMO4*$^{fl/fl}$ and WAP-*Cre-LMO4*$^{fl/fl}$ mice (Figure 1c and d). While we found an increase in apoptosis of mammary epithelial cells at day 13.5 in WAP-*Cre-LMO4*$^{fl/fl}$ mice (data not shown), apoptosis was not increased in the MMTV-*Cre-LMO4*$^{fl/fl}$ mice, leading us to conclude that decreased epithelial cell proliferation is the major cause of decreased lobuloalveolar development in *LMO4* knockout mice. These data add to previous studies (Wang *et al*., 2004; Sum *et al*., 2005c) implicating the *LMO4* gene in lobuloalveolar development, by demonstrating consistent phenotypes in two types of mammary gland knockouts and by showing that LMO4 acts as early as day 5.5 in the pregnant mammary gland.

LMO4 increases apoptosis in MCF-7 breast cancer cells

To start investigating the effect of LMO4 in breast cancer, we selected the human MCF-7 breast cancer cell line for establishing an inducible LMO4 expression system; MCF-7 cells have low LMO4 levels (Wang *et al*., 2004). We used the Tet-off system to establish several distinct MCF-7 cell clones, referred to as MCF7-LMO4-TetOff cells, in which the expression of Myc-tagged LMO4 was repressed by the presence of doxycycline in the medium (Figure 2a). The induced LMO4 level is comparable to the LMO4 expression level found in T47D breast cancer cells (data not shown). We first characterized the effect of LMO4 on proliferation, and found no significant effect of LMO4 upregulation on cell numbers (data not shown). We then investigated whether expression of LMO4 affected apoptosis in MCF-7 cells. Removal of doxycycline significantly increased apoptosis in the MCF7-LMO4-TetOff cells, whereas no effect was observed in vector-transfected cells (Figure 2b, left panel). In time-course experiments, LMO4 increased apoptosis in a time-dependent manner (Figure 2b, right panel). Consistent with results from the apoptosis assay, FACS analysis detected a moderate increase in Annexin V staining, as well as more Annexin V-positive dead cells, in the absence of doxycycline (increased LMO4) than in the presence of doxycycline (low LMO4) (Figure 2c). In addition, elevated expression of LMO4 increased the amount of cleaved caspase-7 as detected by western blotting with antibodies against both uncleaved and cleaved caspase-7 (Figure 2d, left panel). When MCF7-LMO4-TetOff cells were treated with the general caspase inhibitor Z-VAD-FMZ, LMO4 was incapable of inducing apoptosis, indicating that the process was caspase-dependent (Figure 2d, right panel). The effect of LMO4 on apoptosis is not limited to MCF7 cells because similar increase in apoptosis was observed in human mammary epithelial cells (HMEC) transduced with retroviruses expressing LMO4 (Supplementary Figure S4). Together, these results show that LMO4 upregulation can induce caspase-dependent apoptosis in breast cancer cells.

Identification of LMO4-responsive genes

To identify LMO4-responsive genes, we profiled gene expression in three distinct MCF7-LMO4-TetOff cell clones in the presence (low LMO4) and absence (increased LMO4) of doxycycline (Figure 2a). Using a cutoff $P<0.01$ (Baldi and Long, 2001), we found that out of nearly 18 000 expressed probe sets only 111 and 98 were upregulated and downregulated, respectively (Figure 3a). Among the differentially expressed genes, apoptosis is the only Gene Ontology biological process category that is significantly enriched ($P=0.006$; Dennis

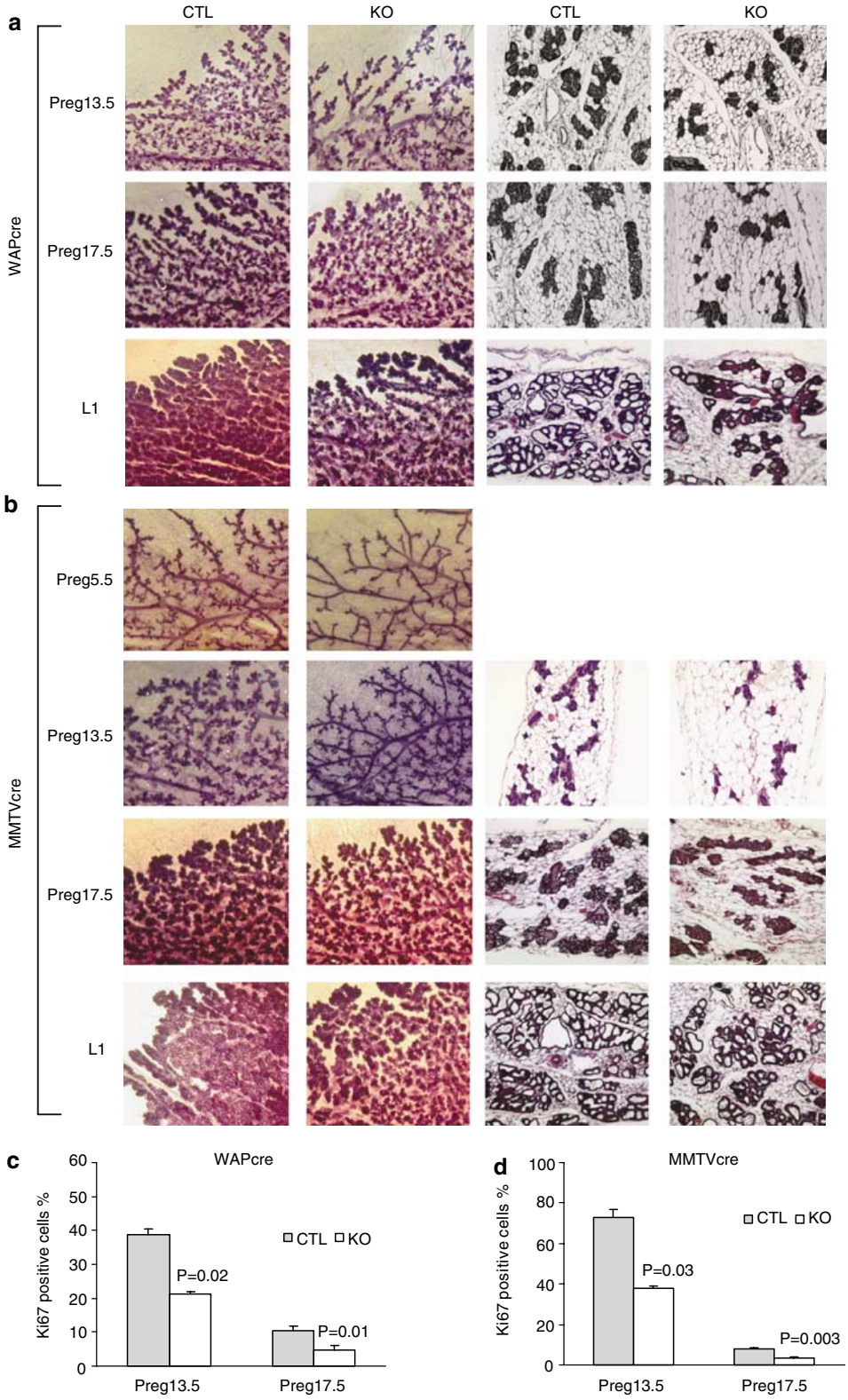

Figure 1 Deletion of the LMO4 gene in mammary glands of mice leads to decreased proliferation of mammary epithelial cells and impaired lobuloalveolar development. (**a**) Whole-mount (first two columns) and histological analyses (last two columns, $\times 100$ magnification) of the fourth inguinal mammary gland from WAP-*Cre-LMO4*$^{fl/fl}$ mice and their controls (WAP-*Cre-LMO4*$^{fl/+}$). (**b**) Whole-mount (first two columns) and histological analyses (last two columns, $\times 100$ magnification) of the fourth inguinal mammary gland from MMTV-*Cre-LMO4*$^{fl/fl}$ mice and their controls (MMTV-*Cre-LMO4*$^{fl/+}$). Quantification of Ki67-positive cells in WAP-*Cre-LMO4*$^{fl/fl}$ (**c**) and MMTV-*Cre-LMO4*$^{fl/fl}$ (**d**) mice along with their controls from the indicated stages; 500 cells were counted in at least three wild-type and knockout mice. KO, knockout; L, lactation; Preg, pregnancy; CTL, control.

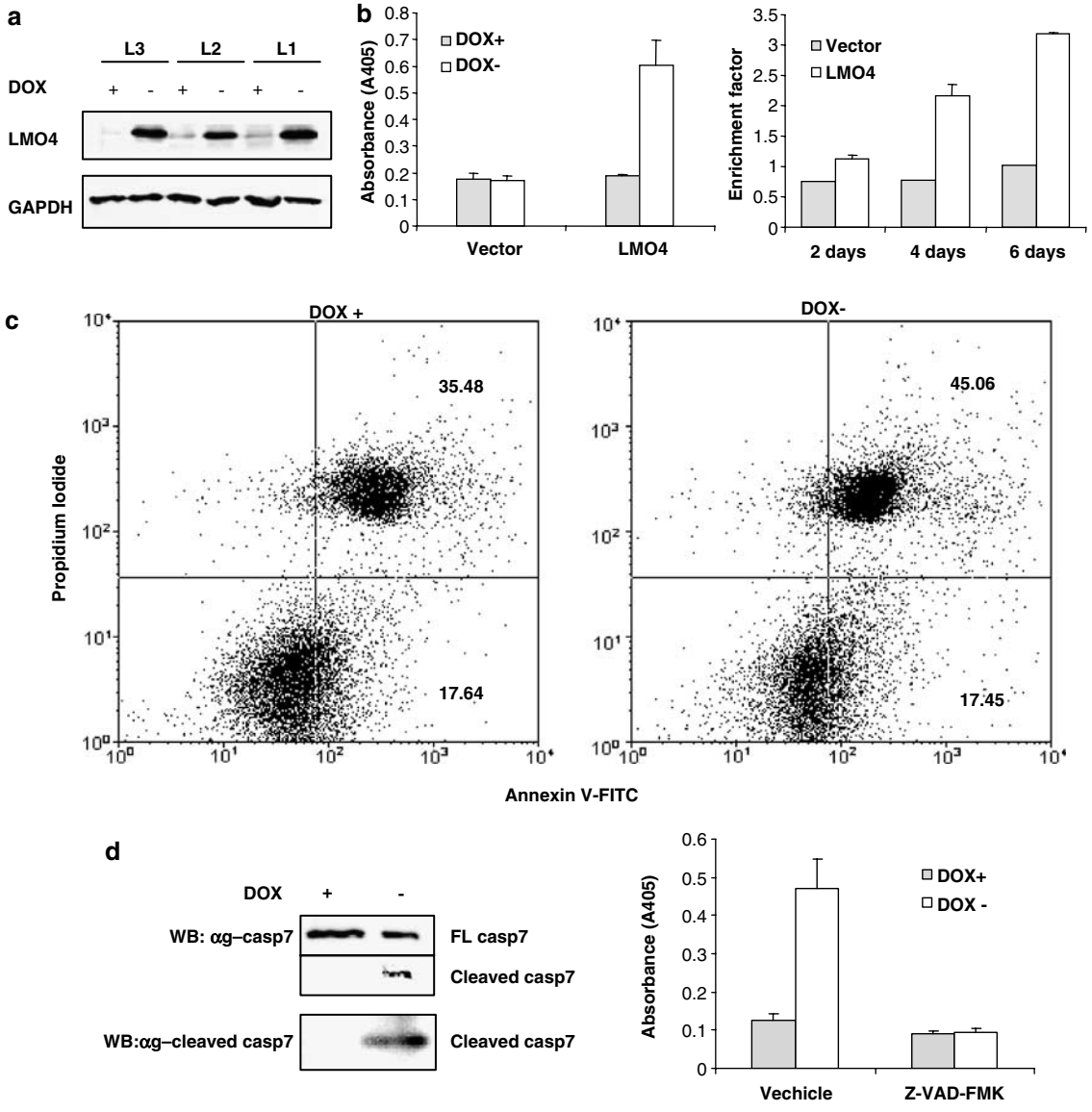

Figure 2 LMO4 expression increases apoptosis in MCF-7 cells. (**a**) Western blot analysis of LMO4 expression in three distinct MCF7-LMO4-TetOff cell clones, L1–3. Extracts from cells treated with (+) and without (−) doxycycline were probed with an antibody to detect the Myc-tagged LMO4 (upper panel) and GAPDH (bottom panel). (**b**) In the left panel, MCF7-LMO4-TetOff cells and MCF-7 cells transfected with vector alone were treated with (filled bars) and without (open bars) doxycycline for 6 days. The right panel shows a time-course study where MCF7-LMO4-TetOff cells (open bars) and MCF-7 cells transfected with an empty vector (filled bars) were treated with and without doxycycline for the indicated times. The enrichment factor is the ratio of apoptosis in cells grown in the absence of DOX to apoptosis in the corresponding control cells grown in the presence of DOX. (**c**) MCF7-LMO4-TetOff cells were grown in the presence and absence of doxycycline for 6 days and analysed with combined propidum iodide/Annexin-V-FITC staining. The numbers in the right bottom and top halves in each panel indicate the percentage of early and late apoptotic cells, respectively. (**d**) In the left panel, MCF7-LMO4-TetOff cells were treated with (+) and without (−) doxycycline for 6 days. Cell lysates were analysed by caspase-7 and cleaved caspase-7 antibodies. In the right panel, MCF7-LMO4-TetOff cells were treated with (filled bars) and without (open bars) doxycycline for 4 days in the presence of vehicle or the caspase inhibitor Z-VAD-FMK. The quantitative data in (**b** and **d**) represent mean and s.e.m. from at least three different experiments.

et al., 2003), consistent with the biological data in Figure 2.

LMO proteins lack DNA-binding domains and regulate gene expression by at least two different mechanisms (Bach, 2000). First, LMOs can recruit LIM-domain transcriptional cofactors Clim/Ldb/Nli to DNA-binding proteins in gene regulatory regions thereby activating transcription. Second, LMOs may act as DN molecules and repress transcription by sequestering Clims away from DNA-binding proteins. Since both models of LMO action are based on interactions with Clims, we reasoned that interfering with Clim function in MCF-7 cells would help identify *bona fide* LMO4-responsive genes. We therefore also created MCF7-DN-Clim-TetOff cell lines where expression of a Myc-tagged dominant-negative (DN) Clim protein is induced upon

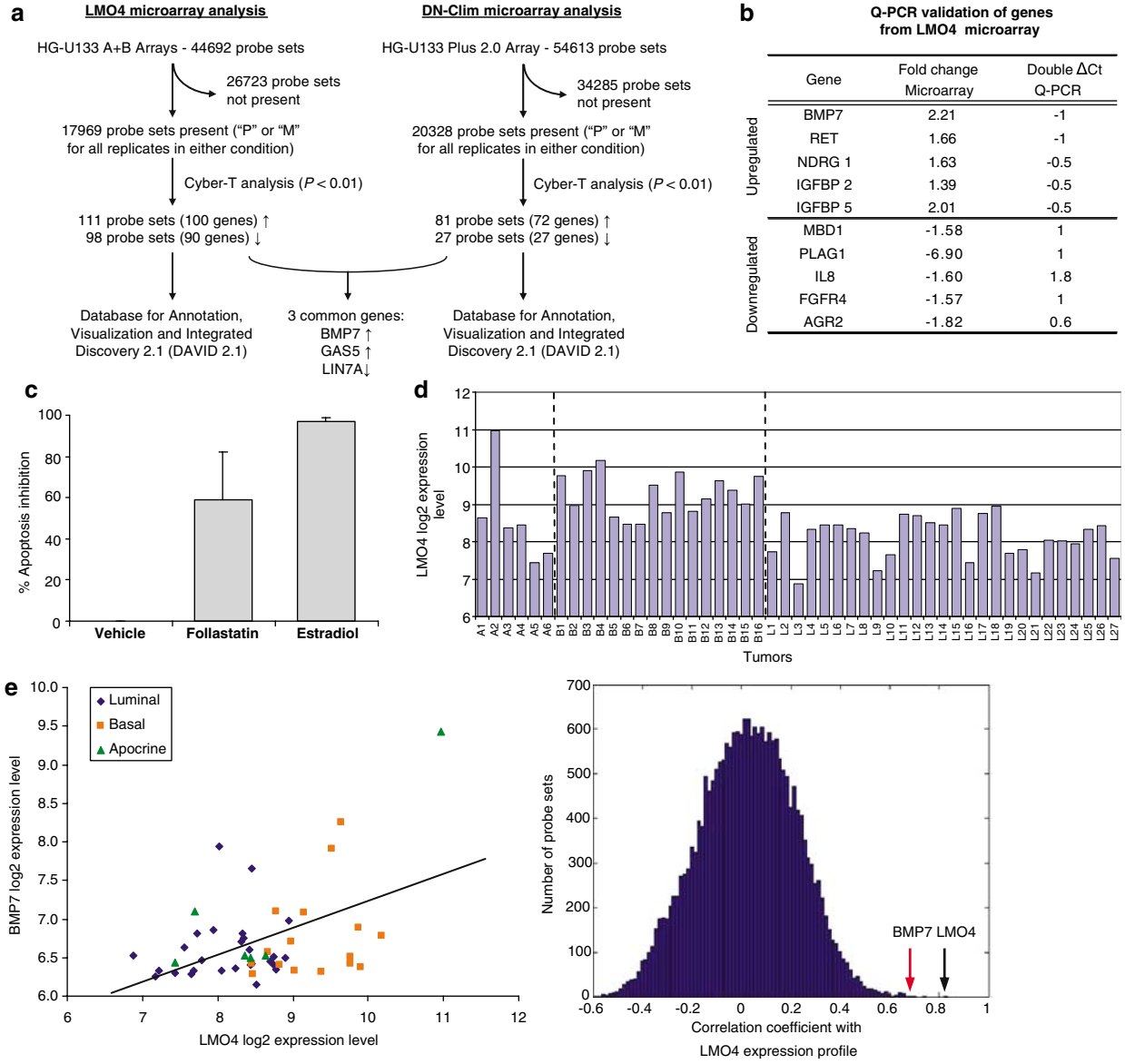

Figure 3 Identification of LMO4 target genes. (**a**) Overview of data processing for microarray gene expression profiling experiments in MCF-7 cells with inducible LMO4 and DN-Clim expression. (**b**) Quantitative real-time PCR validation of LMO4 microarray data. (**c**) Inhibition of apoptosis in MCF7-LMO4-TetOff cells by follistatin and estradiol. Doxycycline was removed from MCF7-LMO4-TetOff cell cultures and 3 days later follistatin (250 ng/ml) or estradiol (20 nM) was added for 3 days. Results represent the mean and s.e.m. from three independent experiments. (**d**) LMO4 transcript levels, shown as log base 2 transformed RMA (robust multichip average) normalized expression levels, in a previous microarray study of 49 individual breast cancer samples (Farmer *et al*., 2005). Broken vertical lines separate the three tumor subtypes defined in this study: A; apocrine, B; basal and L; luminal. (**e**) The left panel shows correlation between LMO4 and BMP7 expression levels in breast tumors. Pearson product moment correlation coefficient, *r*, for the unlogged RMA values is 0.69. The right panel shows distribution of correlation coefficients between the expression of LMO4 and each probe set on the Affymetrix HG-U133A array across all breast tumors. Red arrow, probe set for BMP7. Black arrow, another probe set for LMO4.

doxycycline removal (Supplementary Figure S5). The DN-Clim protein is composed of the C-terminal domain responsible for interactions with LIM domains and other DNA-binding proteins, but lacks the N terminus, which is critical for dimerization and transactivation (Jurata *et al*., 1998). Therefore, DN-Clim blocks productive interactions between LMO4 and Clims.

We then performed microarray analysis in the MCF7-DN-Clim-TetOff cells, comparing expression profiles under control conditions with cells expressing the DN-Clim (Figure 3a); we also found an enrichment of the apoptosis category ($P = 0.049$) in the differentially expressed genes. Interestingly, three genes were significantly differentially expressed ($P < 0.01$) by both LMO4 and DN-Clim: BMP7, GAS5 and LIN7A. All three genes were altered in the same direction in both cell lines, suggesting that in MCF-7 cells, LMO4 upregulation functions by disrupting Clim-containing transcription

complexes on these responsive genes. This is similar to the *Drosophila* wing where LMO4 upregulation and DN-Clim have similar effects (Milan *et al*., 1998). We used quantitative real-time PCR to validate the microarray results for several LMO4-responsive genes, including BMP7 (Figure 3b). Doxycycline had no effect on the expression of these genes in cell clones transfected with vector alone (not shown). For reference, complete lists of differentially expressed genes by LMO4 and DN-Clim with cutoff $P<0.05$ are provided (Supplementary Figures S6 and S7, respectively). All primary microarray data are accessible at www.ncbi.nlm.nih.gov/geo (GSE7382).

We selected BMP7 for further study because BMP7 mediates epithelial–mesenchymal signaling (Dean *et al*., 2004), and epithelial cell apoptosis and proliferation in several different organ systems (Luo *et al*., 1995; Monroe *et al*., 2000). Consistent with these observations, we found that BMP7 can decrease proliferation and increase apoptosis of normal and cancerous mammary epithelial cells (Supplementary Figure S8). In addition, follistatin, an inhibitor of the BMP5-7 subclass (Augsburger *et al*., 1999), decreased LMO4-induced apoptosis of MCF7 cells (Figure 3c), indicating that LMO4-stimulated apoptosis is at least in part mediated by BMPs. Estradiol has been shown to downregulate both BMP7 and its receptor ActRIIB in a variety of hormone-responsive epithelial cells (Kusumegi *et al*., 2004). In fact, estradiol inhibits apoptosis in epithelial cells of reproductive epithelia by suppressing BMP7 signaling (Monroe *et al*., 2000). Interestingly, estradiol completely inhibited LMO4-mediated apoptosis in MCF7 cells (Figure 3c). Together, results from BMP7 and follistatin treatment experiments suggest that BMP7 is a candidate mediator of LMO4 effects in mammary epithelial cells.

To investigate whether there is a correlation between LMO4 and BMP7 levels in human breast cancer, we analysed an expression profiling study on primary breast cancers (Farmer *et al*., 2005). Consistent with a previous report, showing association between high LMO4 expression and ER-negative status of tumors (Gruvberger *et al*., 2001), the average LMO4 expression level is significantly higher (2.21-fold; $P<0.0001$) in basal than in luminal tumors (Figure 3d). In addition, there is a strong correlation ($r=0.69$) between LMO4 and BMP7 expression levels in all tumor subtypes (Figure 3e, left panel). The distribution of correlation coefficients between the expression of LMO4 and each probe set on the Affymetrix array across all breast tumors shows that BMP7 has one of the highest correlation coefficients and is significantly ($P<0.0001$) correlated (Figure 3e, right panel). As expected, the top correlation coefficient is for another LMO4 probe set (Figure 3e, right panel). These results indicate that LMO4 can regulate BMP7 expression in breast cancer cells.

The BMP7 *gene is a direct target of LMO4*

To investigate whether LMO4 directly regulates the BMP7 gene, we cloned 1.9 kb of the proximal 5′ flanking region of the BMP7 gene (Kawai and Sugiura, 2001) upstream of luciferase (pGL3-1.9BMP7). LMO4 was able to upregulate luciferase activity by as much as eightfold (Figure 4a, left panel). These effects were specific because LMO4 had no effect on expression of the unrelated GAL-TK-Luciferase plasmid (data not shown). Further specificity was demonstrated by testing the effect of LMO4 on 5′ deletion mutants of the pGL3-1.9BMP7 plasmid (Figure 4a, right panel). LMO4 was incapable of activating the expression of BMP7 reporter plasmids containing 1.2 and 0.6 kb of proximal 5′ flanking sequence. Together, these results suggest that BMP7 may be a direct target of LMO4 and that the promoter region from −1.2 to −1.9 kb is critical for LMO4 activation of the BMP7 promoter. Interestingly, this region of the promoter has been proposed to contain a repressor element (Kawai and Sugiura, 2001).

To test whether LMO4 binds to *BMP7* promoter, we performed chromatin immunoprecipitation (ChIP) assays in MCF7-LMO4-TetOff cells in the presence and absence of doxycycline. Myc-tag antibody precipitated the *BMP7* promoter in the absence of doxycycline (high LMO4) (Figure 4b, lane 6), indicating that LMO4 associates with the *BMP7* promoter. These results are specific because the *BMP7* promoter was not precipitated by IgG (Figure 4b, lane 5), and the Myc-tag antibody did not precipitate the U6 promoter (Figure 4b, bottom panel). Note that since we used Myc-tag antibody, which only detects the tagged inducible protein, we did not detect the endogenous LMO4 protein in the presence of doxycycline (high LMO4) (Figure 4b, lane 4). To test whether Clim and LMO4 can form a complex on the *BMP7* promoter, we performed double ChIP assays in the MCF7-LMO4-TetOff cells, first precipitating the tagged LMO4 with a Myc antibody and then the endogenous Clim2 protein with a Clim2 antibody. Under conditions of high LMO4 expression, Clim antibody could precipitate the LMO4 complex on the *BMP7* promoter (Figure 4c, lane 6), indicating that both LMO4 and Clim2 bind to the *BMP7* promoter, most likely in a complex given the high-affinity interaction between these proteins (Deane *et al*., 2004; Ryan *et al*., 2006). These results are specific because the Clim2 antibody was not able to precipitate IgG-precipitated *BMP7* promoter (Figure 4c, lane 5). We next tested whether endogenous LMO4 and Clim2 can simultaneously bind to the *BMP7* promoter in T47D breast cancer cells, which express relatively high levels of LMO4 (Visvader *et al*., 2001). In these experiments, where we first precipitated with an LMO4 antibody and then with a Clim2 antibody, both LMO4 and Clim2 associate with the *BMP7* promoter (Figure 4d, lane 3). Together, these results suggest that a transcriptional complex containing LMO4 and Clim2 directly regulates *BMP7* promoter activity.

The histone deacetylase HDAC2 is involved in regulation of BMP7 *by LMO4*

Since both LMO4 upregulation and expression of a DN-Clim molecule lead to increased expression of BMP7,

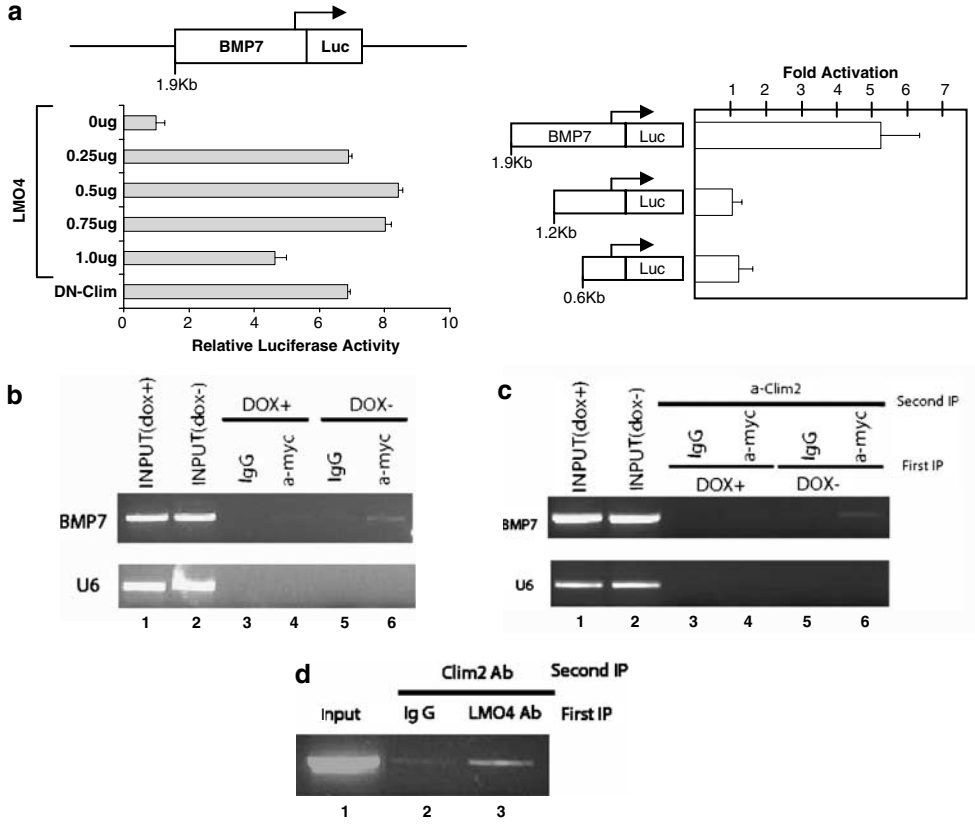

Figure 4 LMO4 and Clim2 associate with and regulate the BMP7 promoter. (**a**) In the left panel, the pGL3-1.9BMP7-Luciferase reporter plasmid was cotransfected into HEK293T cells with the indicated amounts of LMO4 and DN-Clim expression plasmids. In the right panel, the indicated deletion constructs of pGL3-1.9BMP7 were cotransfected with an LMO4 expression plasmid into HEK293T cells. Luciferase activity represents the mean and s.e.m. from at least three independent transfections. (**b**) ChIP assays in MCF7-LMO4-TetOff cells in the presence (DOX+) and absence (DOX−) of doxycycline. (**c**) Double ChIP assays in the MCF7-LMO4-TetOff cells under the indicated conditions. The first antibody was against Myc to precipitate the Myc-tagged LMO4. The second antibody recognized the endogenous Clim2 protein. (**d**) Double ChIP assays in T47D cells. The first antibody recognized the endogenous LMO4 protein and the second antibody recognized the endogenous Clim2 protein.

LMO4 might activate BMP7 by dismissing repressive complexes from the promoter. Therefore, we investigated whether regulated recruitment of histone deacetylases to the BMP7 promoter could account for BMP7 gene regulation by LMO4. First, we performed BMP7 ChIP assays in the MCF7-LMO4-TetOff cells, using specific antibodies against HDAC1–4. Under condition of low LMO4 expression, we clearly detected HDAC2 and HDAC3 association with the BMP7 promoter (Figure 5a, lanes 2 and 3). While HADC3 binding was unchanged, HDAC2 binding to BMP7 promoter was decreased under conditions of high LMO4 expression (Figure 5a, lanes 6 and 7, and b, lanes 3 and 6). These findings suggested that LMO4 might upregulate the BMP7 gene by decreasing recruitment of HDAC2 to the promoter. To test this hypothesis, we performed double ChIP studies, first precipitating LMO4 and then HDAC2 in the MCF7-LMO4-TetOff cells (Figure 5b). Consistent with the model of LMO4 regulation of HDAC2 recruitment, we found that HDAC2 was primarily recruited to the BMP7 promoter under low LMO4 levels (Figure 5b, lanes 3 and 6). As expected, only IP of cells containing high Myc-tagged LMO4 levels revealed recruitment of Clim2 to the promoter in these cells (Figure 5b, lanes 2 and 5). Further support for LMO4 involvement in HDAC2 regulation come from IP experiments where HDAC2 antibody could pull down Myc-tagged LMO4 (Figure 5c), consistent with a recent report also showing that LMO4 can interact with HDAC2 (Singh *et al*., 2005). In transfection assays, an HDAC2 shRNA and HDAC2 expression vector increased and decreased, respectively, expression of the BMP7 promoter (Figure 6a), indicating that HDAC2 suppresses the promoter under basal conditions. Western blot analysis shows effective attenuation of HDAC2 level by HDAC2 RNAi and not by control RNAi (Supplementary Figure S9). When the HDAC2 expression vector was co-transfected with LMO4, it blocked the LMO4-mediated stimulation of the BMP7 promoter (Figure 6a), suggesting that decreased recruitment of HDAC2 could account for LMO4 regulation of the promoter.

Together, this work has identified the *BMP7* gene as a direct target of LMO4. Our findings suggest a novel mechanism for LMO-mediated stimulation of gene expression. According to this model (Figure 6b), a

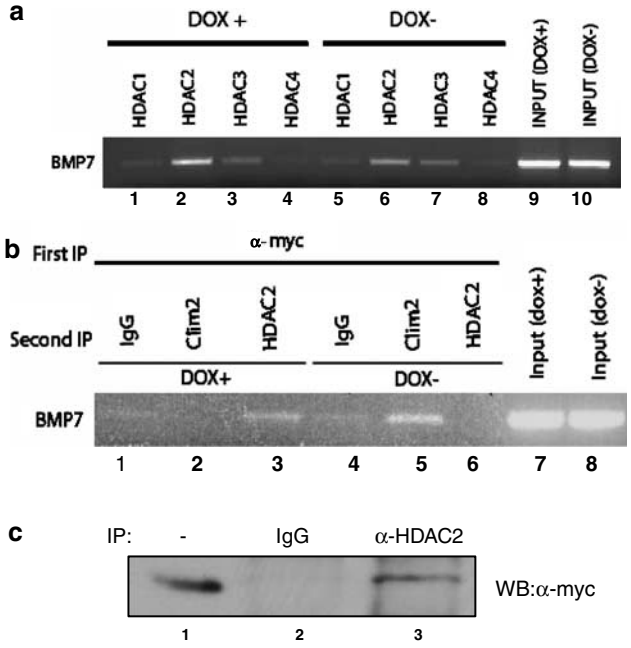

Figure 5 Interactions between LMO4 and HDAC2 on the BMP7 promoter. (**a**) ChIP assays in MCF7-LMO4-TetOff cells in the presence (DOX+) and absence (DOX−) of doxycycline. Antibodies were against HDAC1–4, and primers were the same as in Figure 4b. (**b**) Double ChIP assays in MCF7-LMO4-TetOff cells under the indicated conditions. The first antibody was against Myc-tag to precipitate the Myc-tagged LMO4. The second antibody recognized HDAC2 or Clim2; IgG was used as a negative control. (**c**) IP of MCF7-LMO4-TetOff cell lysates with IgG and HDAC2 antibodies. The Western blot was probed with antibody against Myc to detect the Myc-tagged LMO4.

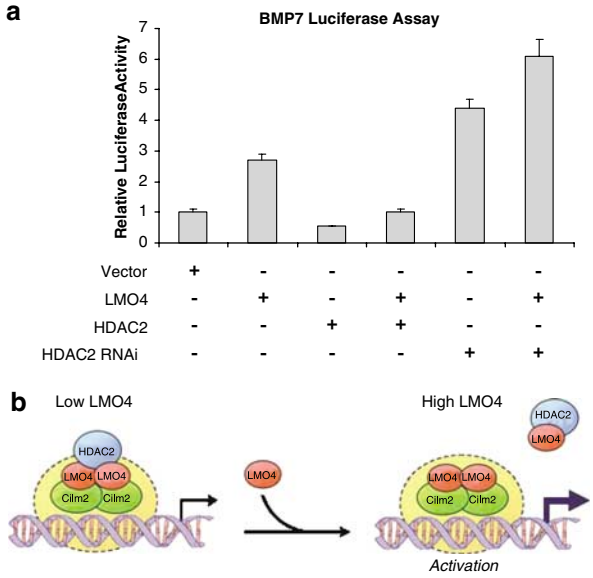

Figure 6 The BMP7 promoter is regulated by LMO4-HDAC2 interactions. (**a**) The PGL3-1.9 BMP7 reporter plasmid was co-transfected with the indicated expression plasmids into HEK293T cells. Luciferase activity represents the mean and s.e.m. from at least three independent transfections. (**b**) A model for activation of the BMP7 promoter by LMO4. The undefined DNA-binding complex with which LMO4/Clim/HDAC2 interact is shown in yellow.

transcription complex containing LMO4 and Clim2 is sensitive to stoichiometry of components such that either overexpression or lowering of LMO4, or expression of DN-Clim, leads to decreased recruitment of HDAC2 to the promoter and activation of transcription.

Discussion

The present study extends previous studies (Wang *et al*., 2004; Sum *et al*., 2005c) by showing, in two distinct types of Cre-mediated mammary gland knockouts of LMO4, that there is decreased proliferation of mammary epithelial cells starting in early pregnancy, ultimately resulting in impaired lobuloalveolar development and mammary gland function at the end of pregnancy. Therefore, one of the roles of LMO4 is to maintain proliferation of mammary epithelial cells during early pregnancy.

A top differentially expressed gene in response to LMO4 expression is *BMP7*, a member of the bone morphogenetic protein family, which has more than 15 mammalian members. Analysis of expression profiles of human breast cancer cases (Farmer *et al*., 2005) showed a highly significant correlation between LMO4 and BMP7 expression levels, indicating that BMP7 could be LMO4 responsive in human breast cancer. The link between LMO4 and BMP7 is especially intriguing because BMPs are secreted cytokines that can control multiple cellular processes, including proliferation, differentiation and apoptosis (Hogan, 1996). By regulating BMP7, LMO4 can have pleiotrophic effects both in cancer and in development. In addition, LMO4 can modulate BMP7 signaling downstream by interacting with Smad8 (Lu *et al*., 2006), one of the effecter Smads that mediates BMP7 signaling. Together, these studies suggest that LMO4 can enhance BMP7 signaling at two different levels.

Given that LMO4 appears to act as a protumorigenic factor, it is interesting that we observed a clear caspase-dependent pro-apoptotic effect in response to LMO4 upregulation in normal mammary epithelial cells and in MCF-7 breast cancer cells. In addition, we found increased apoptosis of mammary epithelial cells in WAP-*Cre-LMO4*$^{fl/fl}$ mice. These findings suggest that either an increase or a decrease in LMO4 levels, perhaps depending on context, may enhance apoptosis. This biphasic effect is similar to findings with several other effectors of apoptosis, including c-myc and ras (Chi *et al*., 1999; Pelengaris *et al*., 2002). In addition, recent studies indicate that the relationship between cell survival and cancer is more complex than thought previously. For example, in the liver system, increased cell death is linked to hepatocarcinogenesis, possibly through compensatory proliferation (Maeda *et al*., 2005). Therefore, it is possible that promotion of apoptosis may be a component of the tumor-promoting effect of LMO4. The effects of LMO4 on cell survival

may also be highly concentration-dependent. For example, we observed a U-shaped dose–response curve between LMO4 levels and Smad-mediated transcription (Lu *et al*., 2006). Finally, it is likely that LMO4 effects are context-dependent; LMO4-mediated signaling to stroma and/or other neighboring cells may be important for the pro-proliferative and pro-tumorigenic effects of LMO4 upregulation *in vivo*. In this regard, the identification of *BMP7* as a target gene of LMO4 is relevant because BMP7 has striking effects on stromal cells (Dean *et al*., 2004), and it is known that growth signaling from stromal fibroblasts during mammary gland development is regulated by TGFβ ligands (Cheng *et al*., 2005).

Like LMO4, BMP7 is highly expressed in the ductular end buds of the developing mammary gland (Sum *et al*., 2005a, b), the site of active proliferation and stromal invasion of the mammary epithelium. LMO4 and BMP7 are also highly expressed in primary breast cancer (Visvader *et al*., 2001; Alarmo *et al*., 2006) and as demonstrated here, their expression is significantly correlated in human breast cancer cases. The fact that BMP7 regulates apoptosis in mammary epithelial cells in the same way as LMO4 is consistent with the idea that it mediates some of LMO4's actions. Interestingly, *LMO4* and *BMP7* knockout mice share common phenotypes, including early postnatal death, malformed sphenoid bones, and fusion of some ribs (Luo *et al*., 1995). The strongest evidence that BMP7 acts downstream from LMO4 comes from studies showing that follistatin, a BMP5-7 blocker, can partially block the pro-apoptotic effect of LMO4 (Figure 3c).

We have identified a novel mechanism for how LMO4 can activate transcription. The histone deacetyl transferase HDAC2 associates with the *BMP7* promoter (Figure 5a and b) and suppresses its activity as demonstrated by both overexpression and loss-of-function experiments of HDAC2 (Figure 6a). It is likely that on the *BMP7* promoter HDAC2 associates with a complex containing LMO4 and Clim2. In support of this possibility, double ChIP experiments for LMO4 and HDAC2 precipitate the BMP7 promoter (Figure 5b), and LMO4 and HDAC2 interact in solution (Figure 5c) (Singh *et al*., 2005). We propose that LMO4 interacts directly with HDAC2, recruiting it to the BMP7 promoter. When LMO4 is upregulated, HDAC2 is sequestered from the promoter, leading to transcriptional activation (Figure 6b).

In summary, we have identified *BMP7* as an LMO4-responsive gene in human breast cancer. Biochemical experiments indicate that *BMP7* is a direct target gene, with LMO4 associating with its promoter and controlling transcription by regulating the recruitment of the histone deacetylase HDAC2. LMO4 and BMP7 have similar effects on proliferation and apoptosis of mammary epithelial cells, and follistatin partially blocks the effect of LMO4, suggesting that BMP7 may at least partially mediate the effects of LMO4 in mammary epithelial cells. These findings are particularly interesting in light of the fact that LMO4 is frequently overexpressed (Visvader *et al*., 2001), especially in ER-negative cases, and is associated with a poor outcome (Gruvberger *et al*., 2001; Sum *et al*., 2005b).

Materials and methods

Generation of mammary gland LMO4 *knockout mice*
B6129-Tg(WAP-*Cre*)11738Mam/J and B6129-Tg(MMTV-*Cre*)4Mam/J mice (Wagner *et al*., 1997) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *LMO4*$^{fl/fl}$ mice on the C57/BL6 background were described previously (Lee *et al*., 2005). We generated mice carrying mammary gland *LMO4* knockouts by mating the WAP-*Cre* and MMTV-*Cre* mice with *LMO4*$^{fl/fl}$ mice. We used published PCR primer sequences for identifying MMTV-*Cre* and WAP-*Cre* mice according to instructions from the Jackson Laboratory. PCR primers for detecting wild type, floxed and deleted *LMO4* alleles are published previously (Lee *et al*., 2005).

Real-time quantitative PCR analysis
Total RNA was extracted with TRIzol® (Invitrogen, Carlsbad, CA, USA) and cDNAs were generated with the High-Capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real-time PCR was performed with SYBR Green or commercially available TaqMan Gene Expression Assays (Applied Biosystems).

Whole-mount mammary gland preparation, histology and immunostaining
The inguinal mammary glands were processed for whole-mount analysis (Wang *et al*., 2004). For histology, mammary glands were fixed in 10% neutral formalin overnight, paraffin embedded, and 6 μm sections were stained with hematoxylin and eosin. To evaluate mammary gland cell proliferation, sections were stained with Ki67 antibody (Novocastra, Norwell, MA, USA) at 1:1000 dilution. We quantified cell proliferation by counting 500 cells in random fields from each mouse and determined the fraction of cells stained with Ki67 antibody.

Cell proliferation and apoptosis assays
Cell numbers were evaluated with the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Apoptosis was quantified with the Cell Death Detection ELISA^{PLUS} kit (Roche), and by Annexin V staining (Roche, Indianapolis, IN, USA; 1858777) and FACS analysis (Lu *et al*., 2006).

Construction of plasmids
We generated the tetracycline-repressible LMO4 and DN-Clim expression plasmids by cloning Myc-tagged LMO4 (Sugihara *et al*., 1998) and DN-Clim (Wang *et al*., 2004) into the pTRE2hyg vector (BD Biosciences, San Jose, CA, USA).

Cell culture and generation of stable cell lines
The MCF7 Tet-Off cell line was obtained from Clontech (Mountain View, CA, USA) (cat 630907) and maintained according to the vendor's recommendations. Normal HMECs were purchased from Cambrex (East Rutherford, NJ, USA), and grown according to the manufacturer's instructions. The pLNCX2 retroviral vectors were previously described (Lu *et al*., 2006).

Microarray analysis
Total RNA was isolated from three distinct MCF7-LMO4-TetOff cell lines and three distinct MCF7-DN-Clim-TetOff cell lines in presence and absence of DOX after 6 days. To decrease variability, we pooled RNAs from three experiments for each of the three cell lines. RNA was labeled and hybridized to Affymetrix DNA chips.

Transient transfection reporter assays
HEK293T cells were seeded into six-well plates 1 day before transfection. Luciferase reporter (1 μg) and effector plasmids (0.5 μg) were co-transfected by calcium phosphate method, and luciferase activity was measured 2 days after transfection as described (Lu *et al*., 2006). All experiments were carried out at least three times, each time in triplicate.

Coimmunoprecipitation and chromatin immunoprecipitations assays
Coimmunoprecipitations (Co-IPs) were performed with extracts from MCF7-LMO4-TetOff cells as described previously (Lu *et al*., 2006). ChIP assays were performed according to the protocol from Upstate Cell Signaling Solutions. Details of antibodies and PCR primers are in Supplementary Information.

Detailed descriptions of Materials and methods can be found in Supplementary Information.

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ORIGINAL ARTICLE

LMO4 can interact with Smad proteins and modulate transforming growth factor-β signaling in epithelial cells

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LIM-only protein 4 (LMO4) plays critical roles in mammalian development, and has been proposed to play roles in epithelial oncogenesis, including breast cancer. As LMO4 is highly expressed in the epithelial compartments at locations of active mesenchymal–epithelial interactions, we reasoned that LMO4 might act by modulating signaling pathways involved in mesenchymal–epithelial signaling. One such candidate signal is the transforming growth factor-β (TGFβ) cytokine pathway, which plays important roles both in development and cancer. We show here that the transcriptional response to TGFβ in epithelial cells is sensitive to LMO4 levels; both up- and downregulation of LMO4 can enhance TGFβ signaling as assessed by a TGFβ-responsive reporter gene. Furthermore, LMO4 can interact with the MH1 and linker domains of receptor-mediated Smad proteins, and associate with the endogenous TGFβ-responsive Plasminogen Activator Inhibitor-1 gene promoter in a TGFβ-dependent manner, suggesting that such interactions may mediate the effects of LMO4 on TGFβ signaling. When introduced into mammary epithelial cells, LMO4 potentiated the growth-inhibitory effects of TGFβ in those cells. These results define a new function for LMO4 as a coactivator in TGFβ signaling, and provide a potential novel mechanism for LMO4-mediated regulation in development and oncogenesis.

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Introduction

LIM-only factor (LMO) 4 belongs to a family of four mammalian LMO proteins (Grutz *et al*., 1998; Kenny *et al*., 1998; Sugihara *et al*., 1998; Racevskis *et al*., 1999); all family members are short transcriptional regulators composed almost entirely of two LIM domains (Bach, 2000). The four LMOs play roles in mammalian development (Yamada *et al*., 1998; Hahm *et al*., 2004; Tse *et al*., 2004; Lee *et al*., 2005). In addition, LMO1 and LMO2 act as oncogenes in acute lymphoblastic leukemia (Rabbitts, 1998), and recent studies have defined LMO3 as an oncogene in neuroblastoma (Aoyama *et al*., 2005) and LMO4 as a protumorigenic factor in breast cancer (Visvader *et al*., 2001; Sum *et al*., 2005b). LMOs interact strongly with transcriptional coregulators referred to as Co-factors of LIM domains (Clims)/LIM domain-binding proteins (Ldb)/nuclear LIM interactors (Nli) (Agulnick *et al*., 1996; Jurata *et al*., 1996; Bach *et al*., 1997, 1999; Visvader *et al*., 1997; Matthews and Visvader, 2003). The Clims also interact with the LIM domains of LIM homeodomain proteins as well as with some transcription factors that lack LIM domains (Torigoi *et al*., 2000; Matthews and Visvader, 2003). Clims, which interact with transcription factors via the C-terminus, are thought to coordinate the assembly of large multiprotein transcriptional complexes through their N-terminally located dimerization domains (Matthews and Visvader, 2003).

LMOs are thought to regulate transcription by several distinct mechanisms. First, by sequestering Clim coregulators participating in gene activation, upregulation of LMOs may repress transcription of genes that are activated by the association of Clims with LIM homeodomain factors (Milan *et al*., 1998; Zeng *et al*., 1998; Milan and Cohen, 2000). Second, LMOs interact with several DNA-binding proteins that lack LIM domains; the best characterized are certain Helix–Loop–Helix and GATA transcription factors (Wadman *et al*., 1994, 1997; de la Calle-Mustienes *et al*., 2003). LMOs are thought to recruit Clim cofactors to such complexes, thereby activating transcription of target genes. Third, because LMOs participate in multiprotein transcription complexes, the stoichiometry of these complexes is critical for transcriptional regulation (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). Coordinated upregulation of LMOs, Clims, and associated DNA-binding proteins may lead to activation, whereas both upregulation and downregulation of individual components may disrupt such complexes. While the levels of LMO4 and Clims are often coordinately regulated during development, in breast

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cancer cells, where LMO4 has been proposed to act in a pro-oncogenic fashion (Sum *et al*., 2005b), LMO4 is often upregulated disproportionately to Clims (Visvader *et al*., 2001; Wang *et al*., 2004).

In addition to neurons, LMO4 is highly expressed in epithelial cells, often at locations of active mesenchymal–epithelial interactions, such as in hair follicles, teeth, epidermis, mammary gland, kidney, and lungs (Sugihara *et al*., 1998; Hermanson *et al*., 1999; Wang *et al*., 2004; Sum *et al*., 2005a). We and others have found that LMO4 can interact with distinct DNA-binding proteins expressed at these locations (Sugihara *et al*., 1998; Sum *et al*., 2002; Kudryavtseva *et al*., 2003; Manetopoulos *et al*., 2003). As LMO4 is highly expressed at multiple sites of mesenchymal–epithelial interactions, it is attractive to propose that LMO4 interacts with and modulates the function of DNA-binding proteins in conserved signaling pathways involved in mesenchymal–epithelial signaling.

The Smad proteins, key mediators of the transforming growth factor-β (TGFβ)/bone morphogenic protein (BMP) superfamily of ligands, provide an example of DNA-binding proteins that play roles in mesenchymal–epithelial interactions in development and cancer (Massague and Wotton, 2000). Smads respond to phosphorylating signals by translocating into the nucleus and associating with target genes as a complex of receptor-activated Smads (R-Smads) and common mediator Smads (Co-Smad; Smad4). Previous work has shown that the Smad transcription complex interacts with several transcription factors, which can positively or negatively modulate TGFβ signal (Derynck and Zhang, 2003). By modulating the binding and activity of Smad proteins on target genes, these Smad-associating proteins are thought to play key roles in TGFβ/BMP signal transduction by affecting the specificity and magnitude of the TGFβ signal in response to environmental effects (Massague and Wotton, 2000).

In this paper, we demonstrate that LMO4 can modulate the proliferative response of epithelial cells to TGFβ signaling. Furthermore, we show that LMO4 interacts with R-Smads and is recruited to genomic Smad-binding sites, suggesting a mechanism for the ability of LMO4 to modulate TGFβ signaling. Our findings link LMO4 to a conserved signaling pathway that plays important roles in epithelial homeostasis.

Results

*LMO4 enhances TGF*β*-mediated transcriptional signal*

LMO4 is upregulated in epithelial cells during the proliferative phase of mammary gland development and in about half of invasive breast cancer cases (Visvader *et al*., 2001; Wang *et al*., 2004). To determine whether LMO4 upregulation could modulate TGFβ signaling, we tested the ability of LMO4 to affect the expression of a well-characterized TGFβ-responsive reporter gene, 9xGAGA-Luciferase (Wieser *et al*., 1995; Dennler *et al*., 1998), which is derived from the regulatory region of the Plasminogen Activator Inhibitor 1 (PAI-1) gene. When the 9xGAGA-Luciferase plasmid was cotransfected with a constitutively active TGFβ receptor 1 (TβR1-AAD) into the kidney epithelial cell line HEK293T, luciferase expression was increased nine-fold (Figure 1a), consistent with previously published data (Dennler *et al*., 1998). Cotransfection of an expression plasmid encoding LMO4 resulted in a dose-dependent expression of LMO4 (Figure 1b) and markedly increased the TβR1-AAD-stimulated luciferase activity, also in a dose-dependent manner (Figure 1a). Moreover, we observed similar enhancing effects of LMO4 on TGFβ1-stimulated 9xGAGA-Luciferase expression in normal human mammary epithelial cells (HMEC) (Figure 1c), and the mouse mammary epithelial cell line NMuMG (Figure 1d). These results indicate that LMO4 can enhance TGFβ-mediated signaling as monitored by the PAI-1 promoter in HEK293T and mammary epithelial cells.

To test whether LMO4 could also modulate the expression of the endogenous PAI-1 gene, we used retroviral transduction to introduce the LMO4 protein into NMuMG cells, and measured PAI-1 mRNA levels with quantitative real-time PCR. Consistent with previous results (Dong-Le Bourhis *et al*., 1998), TGFβ1 increased PAI-1 mRNA expression several fold ($\Delta\Delta C_t = 3$). LMO4 increased PAI-1 mRNA several fold under both basal ($\Delta\Delta C_t = 2.3$) and TGFβ1-stimulated ($\Delta\Delta C_t = 5.7$) conditions (Figure 1e). Taken together, these results suggest that LMO4 upregulation is capable of enhancing TGFβ-stimulated transcription of the PAI-1 gene.

*LMO4 regulates the transcriptional response to TGF*β *in a biphasic manner*

LMO4 regulates transcription by participating in multiprotein complexes that often involve both DNA-binding proteins and other transcriptional coregulators, such as Clims. The stoichiometry of these complexes is critical for their activity and LMO4 upregulation may therefore modulate transcription by disrupting such complexes (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). If this is true, then lowering of LMO4 levels might also lead to changes in gene expression that are similar to those found with LMO4 upregulation; both perturbations, up- and downregulation, would alter the stoichiometry of LMO4-containing transcription complexes. For example, both up- and downregulation of the *Drosophila* Clim homologue, Chip, lead to similar phenotypes in proneural (Ramain *et al*., 2000) and wing (Milan and Cohen, 1999; van Meyel *et al*., 1999) patterning.

To test this idea, we designed three siRNAs against human LMO4 and tested their ability to lower LMO4 levels in T47D breast cancer cells, which express LMO4 at a relatively high level, facilitating the monitoring of endogenous LMO4 protein levels. Of the three LMO4 siRNAs, LMO4 siRNA #1 and #3 effectively decreased endogenous LMO4 levels (Figure 2a; lanes 1 and 3) compared to a negative control siRNA. To test the

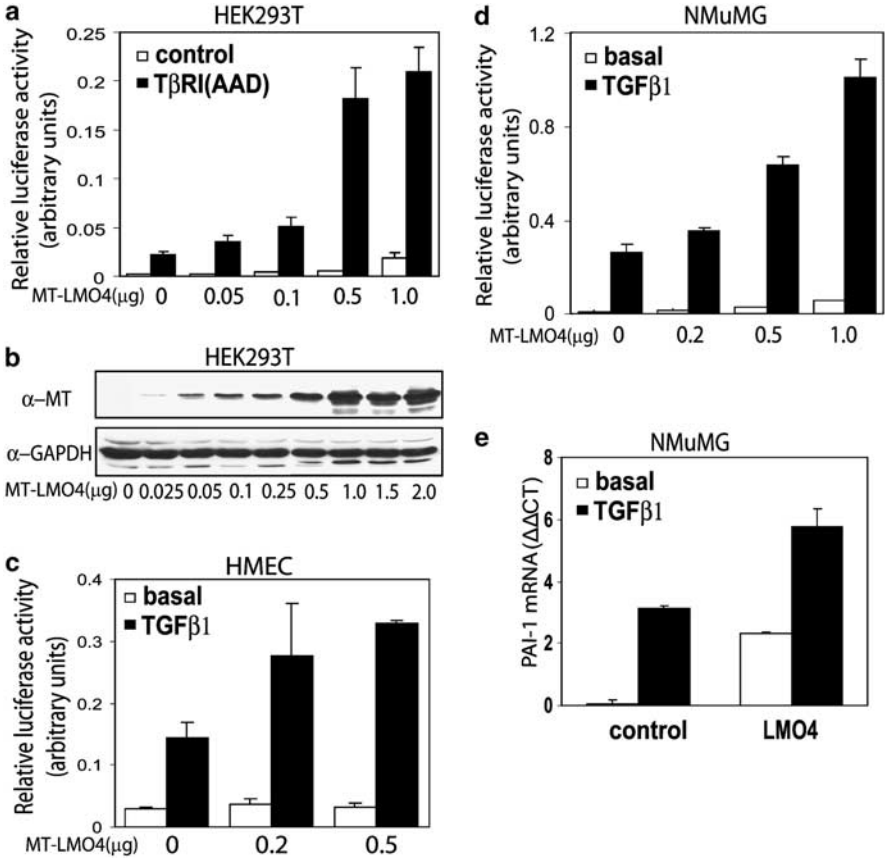

Figure 1 LMO4 potentiates TGFβ-mediated transcriptional activity in epithelial cells. (**a**) The 9xCAGA-Luciferase reporter plasmid (0.5 μg) was transiently cotransfected into HEK-293T cells with either an empty expression plasmid (control) or a plasmid encoding a constitutively activated receptor I of TGFβ (TβRI-AAD; 0.1 μg), which activates TGFβ signaling. An expression plasmid encoding MT-LMO4 was cotransfected in the indicated amounts, ranging from 0 to 1.0 μg; equal amount of DNA was included in all transfections by adjusting the amount of empty expression vector. We determined relative luciferase activity 40 h after the transfection. (**b**) The MT-LMO4 expression plasmid was transfected into HEK293T cells in the indicated concentrations. We isolated whole-cell lysates 40 h later and determined the expression of MT-LMO4 protein by Western blotting with an MT antibody (top panel). As a control for protein concentration and loading, the same blot was also bound to a GAPDH antibody (bottom panel). (**c**) Normal human mammary epithelial (HME) cells were cotransfected with the 9xCAGA-Luciferase reporter plasmid (0.5 μg) and an expression plasmid encoding MT-LMO4 in the indicated amounts. After 24 h, the cells were treated either with vehicle (basal) or TGFβ1 (1 ng/ml) for 20 h before relative luciferase activity was determined. (**d**) Mouse mammary gland (NMuMG) cells were cotransfected with the 9xCAGA-Luciferase reporter plasmid (0.5 μg) and an expression plasmid encoding MT-LMO4 in the indicated amounts. After 24 h, the cells were treated either with vehicle (basal) or TGFβ1 (1 ng/ml) for 20 h before relative luciferase activity was determined. (**e**) NMuMG cells were infected with a retroviruses expressing GFP (control) or LMO4-GFP fusion protein (LMO4). When approximately 80% of the cell monolayers were expressing the target proteins as judged by fluorescent microscopy, the cells were treated either with vehicle alone (basal) or TGFβ1 (1 ng/ml) for 6 h. Total RNA was extracted and endogenous PAI-1 mRNA relative to 18S mRNA levels were determined by real-time PCR. All experiments were carried out in triplicate, and luciferase activity and mRNA levels are expressed as the mean $\pm$ s.d. Similar results were obtained in three different experiments, each one performed in triplicate.

effect of LMO4 siRNA on TGFβ signaling, we transfected into HEK293T cells an expression vector encoding LMO4 shRNA#1 with 9xGAGA-Luciferase reporter plasmid, with and without a TGFβ activator. While the control shRNA had little effect on TGFβ stimulation of reporter activity, the LMO4 shRNA markedly enhanced TGFβ stimulation (Figure 2b). The effect of the LMO4 shRNA was specific because the expression vector that encodes mouse LMO4, which is not targeted by the shRNA, could partially reverse the stimulatory effect of LMO4 shRNA (Figure 2c). As predicted from the experiments described previously (Figure 1), higher amounts of transfected LMO4 ultimately resulted in stimulation of gene expression, creating a U-shaped dose–response curve for the effect of LMO4 on TGFβ-stimulated gene expression (Figure 2c).

Together, these experiments show that in this system, TGFβ signaling is sensitive to LMO4 levels. Very high or low concentration of LMO4 can enhance TGFβ-dependent transcription of the PAI-1 gene reporter. These findings are consistent with results from other systems, showing that the stoichiometry of the components of transcription complexes involving LIM domain transcription factors is critical for regulation of gene activation (Milan and Cohen, 1999; van Meyel *et al*.,

1999; Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003; Matthews and Visvader, 2003).

LMO4 interacts with several R-Smads

TGFβ regulates transcription of the PAI-1 gene by facilitating the nuclear translocation and DNA binding of a complex composed of R-Smads (Smad2 and/or Smad3) and the co-Smad, Smad4 (Massague and Wotton, 2000; Derynck and Zhang, 2003). To investigate the mechanisms of action for the effect of LMO4 on TGFβ-mediated transcription, we tested whether LMO4 could interact with these key mediators of TGFβ-regulated transcription. An expression vector encoding myc-tagged LMO4 was transfected into HEK293T cells with or without HA-tagged Smad1, Smad2, Smad4, and Smad5. Whole-cell extracts were isolated and immunoprecipitated with an myc-tagged antibody followed by SDS gel electrophoresis and immunoblotting with an HA antibody. Smad1, Smad2 and Smad5 were all clearly co-immunoprecipitated with LMO4 (Figure 3a; top panel), suggesting that LMO4 is capable of interacting with several Smad proteins. A weak interaction was also detected between LMO4 and the co-Smad, Smad4 (Figure 3a; lane 5). LMO4 was also co-immunoprecipitated with a Smad2 antibody in non-transfected HEK293T cells (Figure 3b), indicating interaction of endogenous LMO4 and Smad2 proteins.

To validate the co-immunoprecipitation results, and to test whether the LMO4–Smad interactions are direct, we performed GST pull-down assays. We found that LMO4 clearly interacts with Smad2, Smad3, Smad5, and Smad8, with the strongest LMO4 interactions detected with Smad8 (Figure 4a). Consistent with the co-immunoprecipitation results, a weak LMO4 interaction was also detected with Smad4. To map the Smad domains that are responsible for interactions with LMO4, we tested the interactions of LMO4 with subregions of the Smad3 protein. Smad proteins are composed of an N-terminal Mad homology (MH) domain 1, which is responsible for nuclear import and DNA binding, except in the case of the major splice form of Smad2, which contains an insertion in these regions and does not directly bind DNA. A C-terminal MH2 domain, which mediates Smad oligomerization, is linked to the MH1 domain with a less-conserved linker domain (Massague and Wotton, 2000; Derynck and Zhang, 2003). All three domains have been shown to interact with several transcription factors as well as cytoplasmic adaptors (Massague and Wotton, 2000; Derynck and Zhang, 2003). In these experiments, LMO4 interacted with the MH1 and linker domains of Smad3; no interaction was found with the MH2 domain (Figure 4b).

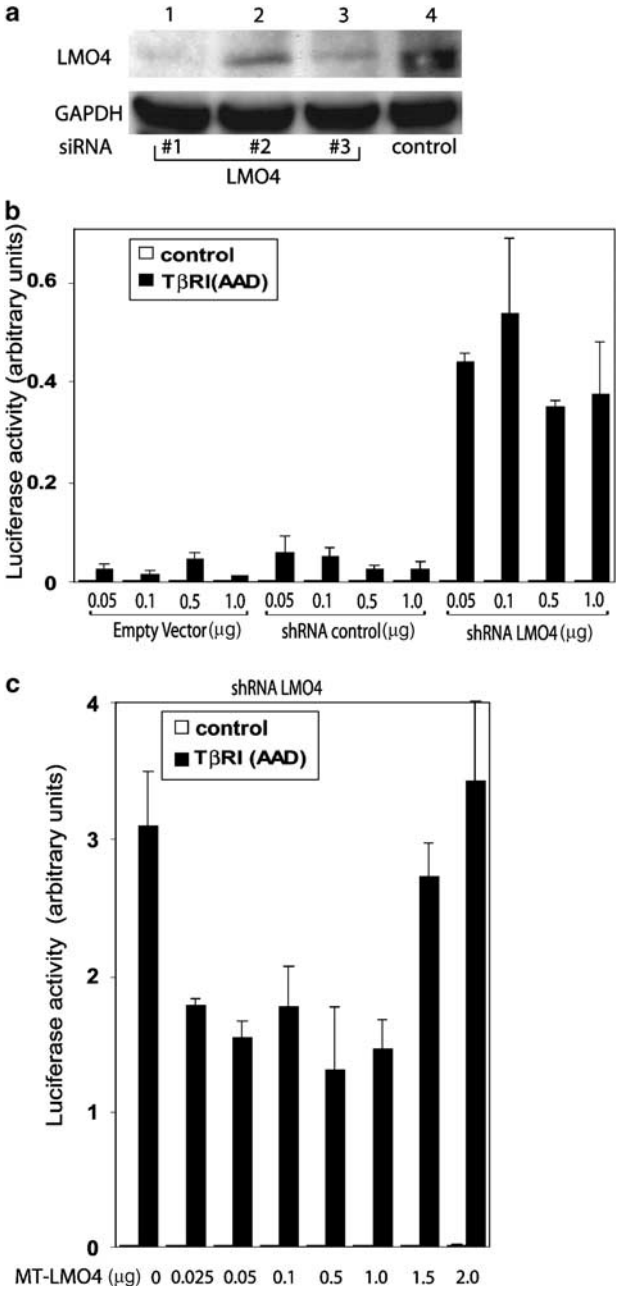

Figure 2 Biphasic regulation of PAI-1 reporter activity by LMO4. (**a**) Three distinct siRNAs targeting human LMO4 and a control siRNA were transfected into T47D breast cancer cells, using RNAiFect transfection reagent (Qiagen). After 40 h, LMO4 protein levels were determined by Western blotting of whole-cell lysates with LMO4 antibody (top panel). As a control, the same blot was bound to GAPDH antibody (bottom panel). (**b**) HEK293T cells were cotransfected with the 9xCAGA-Luciferase construct (0.5 μg) and either an empty expression plasmid (control) or a plasmid encoding a TGFβ activator (TβRI-AAD; 0.1 μg). To test the effect of lowering LMO4, we also transfected the indicated amounts of empty shRNA expression vector, control shRNA expression vector, and LMO4 shRNA expression vector. (**c**) HEK293T cells were cotransfected with the 9xCAGA-Luciferase construct (0.5 μg) and either an empty expression plasmid (control) or a plasmid encoding a TGFβ activator (TβRI-AAD; 0.1 μg). In addition, the vector expressing human LMO4 shRNA#1 (0.5 μg) was included under all conditions. An expression vector that encodes mouse MT-LMO4 in the indicated concentrations was cotransfected. At 40 h after transfection, luciferase activity was determined; relative luciferase activity is expressed as the mean ± s.d. from triplicate transfection. Similar results were obtained in three independent experiments.

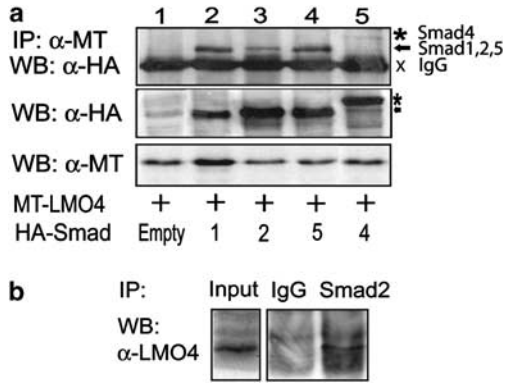

Figure 3 LMO4 interacts with several Smad proteins. (**a**) MT-tagged LMO4 and HA-tagged Smad1 (lane 2), Smad2 (lane 3), Smad4 (lane 5), and Smad5 (lane 4) were cotransfected into HEK293T cell. At 2 days after transfection, whole-cell lysates were isolated. Cell lysates were immunoprecipitated with anti-MT and the Smad proteins in the complex identified with immunoblotting with anti-HA (top panel). Smad and LMO4 protein expression was demonstrated with direct immunoblotting of cell lysates with HA antibody (middle panel) and MT antibody (bottom panel), respectively. The asterisk indicates the location of Smad4, the arrow the location of Smad2, -3 and -5, and the X the location of IgG. (**b**) Lysates from HEK293T cells were immunoprecipitated with either IgG or Smad2 antibody, and immunoblotted with an LMO4 antibody.

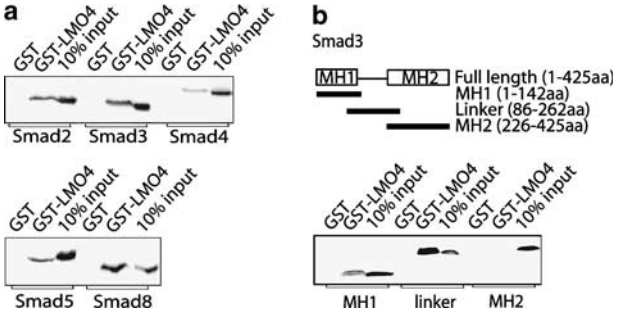

Figure 4 LMO4 interacts with the MH1 and linker regions of Smad proteins. (**a**) Full-length, ^{35}S-labeled Smad2, Smad3, Smad4, Smad5, and Smad8 were incubated with either GST alone or GST-LMO4. LMO4–Smad interactions were determined with GST pull-down assays and compared to 10% of the Smad protein input as visualized by SDS–PAGE and autoradiography. (**b**) GST pull-down assays were used to determine interactions between GST-LMO4 and the indicated ^{35}S-labeled subdomains of Smad3.

These data suggest that LMO4 may modulate the transcriptional response to TGFβ by interacting with Smad proteins, and that both the MH1 and linker domains of Smad3 participate in the interaction.

LMO4 can associate with the PAI-1 endogenous promoter in vivo *in response to TGF*β

During TGFβ signaling, R-Smads are phosphorylated by the activated receptor and form complexes with the co-Smad Smad4, after which the R-Smad/Smad4 complex enters the nucleus and associates with target genes (Massague and Wotton, 2000). To test whether LMO4 affects the phosphorylation of R-Smads, HEK293T cells were transfected with a control vector or LMO4, followed by treatment with vehicle or TGFβ1. We assessed the phosphorylation of endogenous Smad2 by Western blotting with an antibody recognizing phosphorylated Smad2. LMO4 had no effect on TGFβ1-induced Smad2 phosphorylation (Supplemental Figure 1A). To test whether LMO4 affects the R-Smad–Smad4 interaction, a Flag-tagged Smad3 and an HA-tagged Smad4 were cotransfected into HEK293T cell with or without MT-LMO4. After TGFβ1 treatment, the interaction between Flag-Smad3 and HA-Smad4 was analysed with immunoprecipitation and Western blotting. While TGFβ1 markedly enhanced Smad3/Smad4 complex formation, LMO4 had no effect on the complex formation (Supplemental Figure 1B). Together, these results suggest that LMO4 affects TGFβ signaling downstream of R-Smad phosphorylation and R-Smad/Smad4 complex formation. Based on these experiments and the protein–protein interaction results (Figures 3 and 4), we hypothesized that LMO4 might associate with Smad complexes on target genes.

To test whether LMO4 can associate with the PAI-1 promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays. HEK293T cells, untreated or treated with TGFβ1, were transfected with an empty vector or expression vectors encoding MT-Smad4 or MT-LMO4. ChIP assays were performed as previously described using myc(MT) antibodies with binding to the endogenous PAI-1 promoter detected with PCR using specific oligonucleotides (Kurisaki *et al*., 2003). As expected, Smad4 associates with the PAI-1 promoter, with binding greatly increased after TGFβ1 treatment (Figure 5a; lanes 1 and 2). Interestingly, LMO4 also associates with the PAI-1 endogenous promoter in a TGFβ1-dependent manner (Figure 5a; lanes 4 and 5), consistent with its ability to interact with Smad proteins and regulate the PAI-1 promoter. The MT antibody is specific in this assay because the PAI-1 promoter was not precipitated in cells transfected with an empty vector (Figure 5a; lane 3), and nonspecific IgG did not precipitate the PAI-1 promoter (Figure 5b; lanes 1–4) in an experiment where LMO4 associated with the promoter in a TGFβ1-dependent manner (Figure 5b; lanes 5 and 6). The association of LMO4 to the PAI-1 regulator, region is also promoter specific because no binding was detected to the GAPDH promoter (Figure 5c), which is regulated neither by TGFβ nor LMO4. Taken together with the results from transient transfection assays and protein–protein interaction studies, these data suggest that LMO4 can bind the PAI-1 promoter in a TGFβ-dependent fashion. This may occur via direct association with Smad proteins, resulting in modulation of promoter activity.

*LMO4 potentiates TGF*β*-mediated inhibition of cell proliferation*

Among the many different effects of TGFβ, inhibition of epithelial cell growth, either by suppression of cell proliferation or enhanced apoptosis, is one of the best-characterized (Derynck *et al*., 2001). Therefore, to test

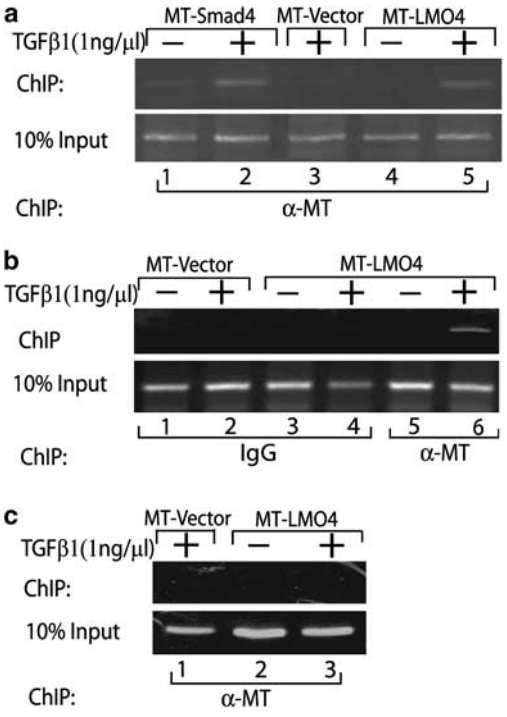

Figure 5 LMO4 associates with the endogenous PAI-1 promoter in a TGFβ-dependent fashion. (**a–c**) HEK293T cells grown in 100 mm dishes were transfected with 2 μg of empty expression vector or the same amount of expression vectors encoding MT-LMO4 or MT-Smad4, using Lipofectamine 2000. On the third day after transfection, cells were treated with vehicle or TGFβ1 (1 ng/ml) for 2 h. LMO4-associated DNA was isolated by ChIP with anti-MT or normal mouse IgG as a negative control, followed by PCR with primers specific for the PAI-1 promoter (**a** and **b**) or the GAPDH promoter (**c**). As a control, 10% of the input DNA was also PCR-amplified (lower panels in **a**, **b**, and **c**).

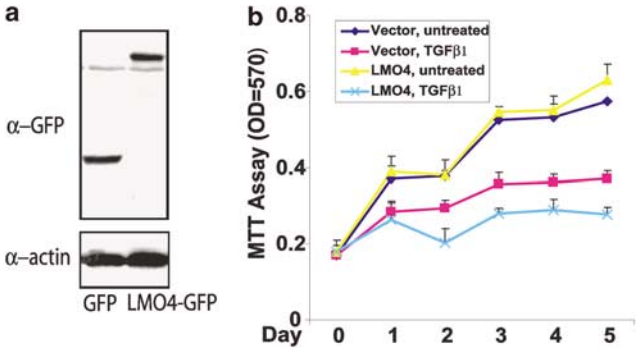

Figure 6 LMO4 enhances the inhibitory effect of TGFβ on human mammary epithelial cell growth. (**a**) HME cells were infected with equivalent pfu of retroviruses encoding GFP alone or LMO4-GFP. After 2–3 rounds of infections, about 80% of HME cells were expressing the target proteins as assessed by immunomicroscopy (not shown). At that time, whole-cell lysates were isolated and analysed by Western blotting with GFP antibody (top panel). As a control, the same blot was also bound to actin antibody (bottom panel). (**b**) HME cells expressing either LMO4-GFP or the control protein GFP were plated onto 96-well plates (5000 cells/well). After treatment with TGFβ1 (1 ng/ml) for 24 h, cells were grown in fresh grow medium for another 4 days; cell growth was monitored, using the MTT assay. MTT assays were performed in 10-replicate determination and results are expressed as the mean $\pm$ s.d. at OD = 570 nm. Three independent experiments were performed; the data from a representative experiment are shown.

whether LMO4 can modulate the *in vivo* function of TGFβ signaling, we introduced viral vectors expressing either green fluorescent protein (GFP) or LMO4-GFP fusion proteins into normal HMEC. Expression from the GFP and LMO4-GFP vectors was equivalent in these experiments (Figure 6a) and for both vectors about 80% of cells expressed the proteins as determined by the GFP signal (data not shown). Cells were treated either with vehicle or TGFβ1 for 24 h and their growth was monitored over the course of 5 days, using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. As expected, TGFβ1 inhibited the growth of HMEC in a time-dependent manner (Figure 6b). Interestingly, LMO4 significantly potentiated the cytostatic effect of TGFβ1 (Figure 6b). In contrast, LMO4 had no significant effect on the growth of untreated HMEC (Figure 6b).

To test whether the effect of LMO4 on the growth of HMEC was due to inhibition of proliferation or increased apoptosis, we first examined the effect of LMO4 on proliferation of HMEC, using the 5-(and 6-) carboxy fluoroscein diacetate succimidyl ester (CFSE) assay. As expected, TGFβ1 inhibited the proliferation of HMEC in a time-dependent fashion (Figure 7a; top panels). The introduction of LMO4 by retroviral transduction inhibited proliferation of HMEC (Figure 7a; middle panels). Expression from the control vector (TAP) and the vector expressing LMO4-TAP was similar (Figure 7b). To test whether cell death was modulated by LMO4, we monitored apoptosis after introduction of LMO4 in the presence and absence of TGFβ1 in HMEC, using Annexin V staining in combination with FACS analysis. TGFβ1 treatment increased the fraction of apoptotic HMEC from 6.43 to 11.21% and this effect was not significantly modulated by LMO4 (Figure 7c), suggesting that LMO4 does not alter the growth of HMEC by affecting apoptosis. Together, these experiments suggest that LMO4 affects cell growth by potentiating the inhibitory effect of TGFβ on cell proliferation.

In summary, our results suggest a novel function for LMO4 in TGFβ signaling. Based on our findings, we propose a model in which LMO4 interacts with Smad proteins on target genes, thereby modulating the cytostatic response of TGFβ.

Discussion

In this manuscript, we provide new information that the transcriptional coactivator LMO4 can modulate the cytostatic effects of TGFβ in epithelial cells. Using ChIP and transient transfection transcription assays, we demonstrate that LMO4 can associate with and regulate a prototype Smad target promoter.

One of the striking features of TGFβ signaling is the pleiotropic nature of its biological effect (Massague and

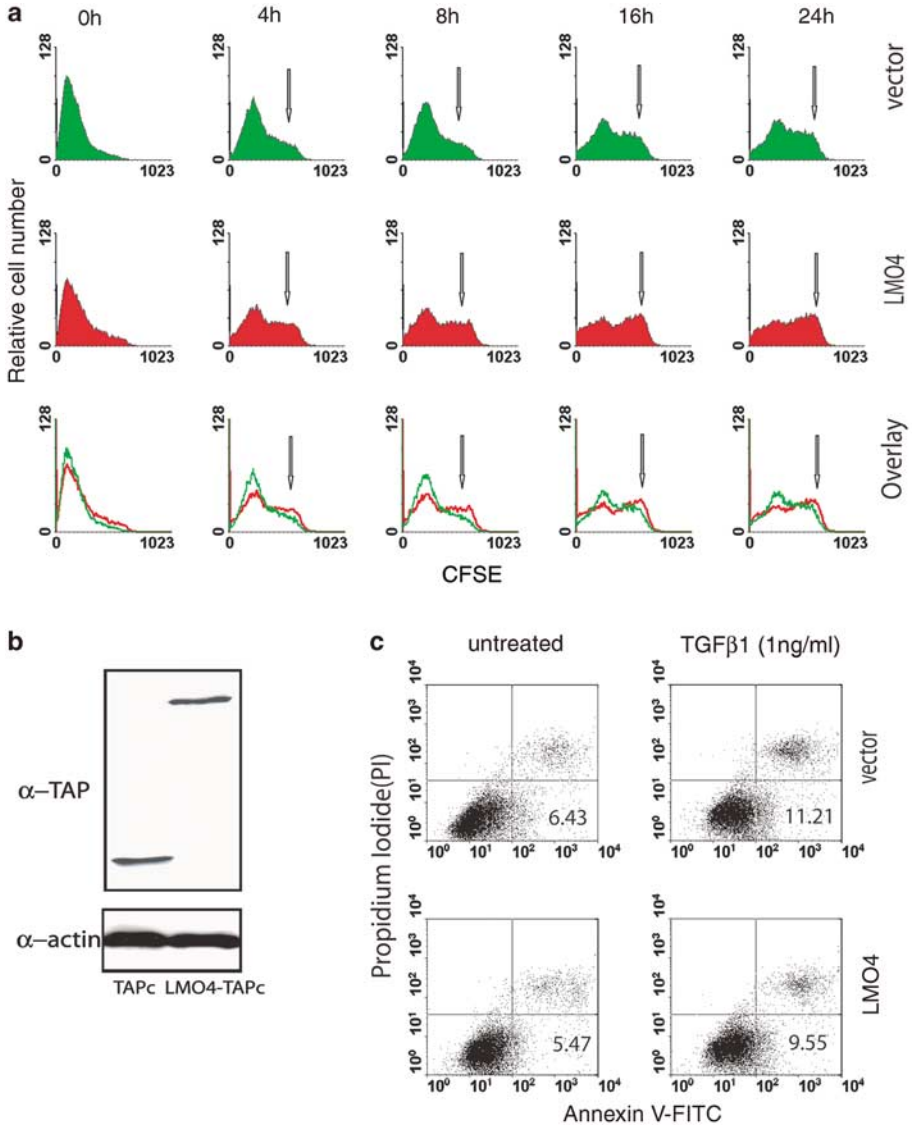

Figure 7 LMO4 enhances the inhibitory effect of TGFβ on HME cell proliferation, but has no effect on TGFβ-induced apoptosis. (**a**) HME cells were infected with retroviruses encoding LMO4-TAPc fusion protein or TAP alone as described for the experiment in Figure 6. HME cells expressing either control protein TAP (top panel) or LMO4-TAPc (middle panel) were stained with CFSE and then plated onto six-well plates (10 000 cells/well). On the second day, cells were treated with TGFβ1 (1 ng/ml) for the indicated time, and then grown in fresh medium for another 3 days. Cell proliferation was assessed with a FACS based on CFSE quantity. The CFSE amount in a single cell will decrease by 50% with each cell division. The arrows point to cells that contain large amount of CSFE, indicating slow proliferation. The third panel contains overlay of the TAPc (vector) and LMO4-TAPc (LMO4) panels and shows the relative abundance of slow-growing cells in the LMO4-infected panel. The data from a single representative experiment (out of three) are shown. (**b**) Expression of TAP and LMO4-TAPc in HME cell lysates was assessed by immunoblotting with TAP antibody (top panel). As a control, the same blot was also analysed by an Actin antibody (bottom panel). (**c**) HME cells expressing either TAP control protein or LMO4-TAPc were seeded onto 60-mm dishes (1×10^5 cells/dish). The next day, cells were treated with either vehicle (untreated) or TGFβ1 (1 ng/ml) for 24 h. Cell apoptosis was analysed with combined propidium iodide/annexin-V-FITC staining. The number in right-bottom half in each panel indicates the percentage of apoptotic cells. Similar results were obtained from three different experiments.

Wotton, 2000; Derynck and Zhang, 2003). Depending on context, TGFβ can selectively regulate proliferation, apoptosis, migration, epithelial–mesenchymal transition, as well as other cellular features. In addition, the effects of TGFβ are highly dependent on the responding cell type. Our data add to the growing literature suggesting that interactions of Smad proteins with other transcription factors may, at least in part, underlie the specificity of the multitude of TGFβ actions. Thus, our data suggest that LMO4 has selective effects on TGFβ actions because it modulates cell proliferation (Figures 6 and 7), but has no effect on apoptosis (Figure 7) and epithelial–mesenchymal transition (data not shown). Also, since LMO4 expression is restricted to epithelial cells, our findings suggest one mechanism whereby TGFβ effects are selectively modulated in distinct cell types.

Interestingly, our data predict that within the same cell type, changes in LMO4 levels may either increase or decrease TGFβ signaling, depending on the levels of LMO4 under the basal condition and the magnitude of LMO4 change (Figure 2c). For example, under conditions of very low LMO4 levels, moderate increases in LMO4 may lead to decreased TGFβ effect. However, under conditions of higher basal levels of LMO4, a further increase may enhance TGFβ effect. Smad proteins participate in multiprotein complexes that include transcriptional coactivators and corepressors, as well as DNA-binding proteins (Massague and Wotton, 2000; Derynck and Zhang, 2003). Since both upregulation and downregulation of LMO4 can lead to potentiation of TGFβ activation of the PAI-1 promoter, it is tempting to speculate that LMO4 helps to coordinate complexes on the PAI-1 gene, and that the stoichiometry of the components of these complexes is important. In such a case, both removal and excess of LMO4 is predicted to disrupt multiprotein complexes (Ramain *et al*., 2000; Thaler *et al*., 2002; Lee and Pfaff, 2003). Our findings are consistent with data in *Drosophila* showing that either upregulation or downregulation of the Clim homologue Chip leads to similar developmental phenotypes (Ramain *et al*., 2000).

Our data, which suggest that TGFβ regulation of at least some genes may be sensitive to LMO4 levels, are likely to have implications for understanding LMO4-mediated gene regulation because LMO4 is highly regulated under a variety of conditions that include normal and cancer development, as well as in response to physiological stimuli (Hinks *et al*., 1997; Wang *et al*., 2004). Owing to the cell- and developmental-specific regulation of LMO4, our findings may provide a mechanistic basis for aspects of cell-type- and context-specific gene regulation by TGFβ. Our results, showing that LMO4 overexpression enhances TGFβ-mediated cytostasis, may seem to contradict recent studies, which indicate that LMO4 overexpression promotes tumorigenic properties of mammary epithelial cells (Visvader *et al*., 2001; Sum *et al*., 2005b). However, there are at least two potential explanations for this apparent contradiction. First, because of the U-shaped TGFβ response curve to LMO4 (Figure 2c), the starting point will determine whether LMO4 potentiates or decreases TGFβ signaling; LMO4 overexpression in tumors may inhibit TGFβ signaling. Second, in addition to a direct cytostatic effect, TGFβ has direct and indirect protumorigenic effects; it is possible that LMO4 potentiates the protumorigenic effects of TGFβ *in vivo*.

A striking feature of LMO4 gene expression is its prominent expression in epithelial cells at locations of active reciprocal mesenchymal–epithelial interactions (Sugihara *et al*., 1998; Hermanson *et al*., 1999; Wang *et al*., 2004; Sum *et al*., 2005a). In such organs, including the developing hair follicles, teeth, mammary gland, lungs, and kidneys, BMP signaling has been shown to be very important (Arias, 2001; Waite and Eng, 2003). While our study has focused on the role of TGFβ signaling, it is quite possible that LMO4 could also modulate BMP signaling because we found that it interacts with Smad1, Smad5, and Smad8, which are primarily responsible for mediating BMP signals (Derynck and Zhang, 2003). In this respect, a recent study that used the yeast two-hybrid assay to screen for Smad8-interacting proteins identified LMO4 as a Smad8 partner (Colland *et al*., 2004). This is consistent with our findings that of all Smads tested, the strongest interaction was found between LMO4 and Smad8. This study also showed that LMO4 siRNA could inhibit BMP-7-stimulated transcription of a BMP-responsive reporter gene and the alkaline phosphatase gene in HepG2 cells (Colland *et al*., 2004). Yet, another potential link between LMO4 and BMP signaling comes from studies in *Xenopus* where it was shown that xLMO4 transcripts in ventral mesoderm and the neural plate are upregulated by BMP-4 (de la Calle-Mustienes *et al*., 2003). Functional studies indicate that xLMO4 plays roles in ventral mesoderm identity and neural plate regionalization. Thus, depending on the context, LMO4 may be both induced by BMP signaling and a modulator of the transcriptional effects of BMPs.

Many of the experiments in our study, including the ChIP experiments, were performed with exogenously expressed LMO4. However, it is important to note that we provide strong support for the potential role of endogenous LMO4 in TGFβ signaling. First, we demonstrated an interaction between endogenous LMO4 and Smad2 proteins, suggesting that LMO4 and Smad2 can interact *in vivo* at normal cellular concentrations (Figure 3b). Second, we showed that RNAi-mediated knockdown of LMO4 affected TGFβ signaling, supporting an *in vivo* role for endogenous LMO4 in TGFβ signaling (Figure 2).

For unknown reasons, LMO4 knockout mice die during later stages of embryogenesis or perinatally (Hahm *et al*., 2004; Tse *et al*., 2004; Lee *et al*., 2005). While a significant portion of these mice show exencephaly, even mice without this abnormality die perinatally. In addition, LMO4 knockout mice have skeletal patterning defects involving the basal skull, vertebrae, and ribs. Other homeotic transformations such as fusions of cranial nerves IX and X and defects in cranial nerve V were also observed (Hahm *et al*., 2004). No mice deleted for genes encoding TGFβ superfamily ligands phenocopy all aspects of the LMO4 knockout mice. However, strikingly, mice deleted for the TGFβ2 gene show defects in the sphenoid bone highly similar to those found in LMO4 mutant mice, including a missing presphenoid body; TGFβ2 knockout mice also exhibit rib cage abnormalities similar to the LMO4 knockout mice (Sanford *et al*., 1997). As in the LMO4 knockout mice, skeletal defects of the basal skull, vertebrae, and ribs are prevalent in BMP7 gene-deleted mice (Luo *et al*., 1995). These skeletal abnormalities include rib cage abnormalities that are common to the two, such as misalignment of the ribs on the sternum. Deletion of the BMP antagonist Noggin leads to altered patterning of somites and the neural tube in the mouse, including neural tube closure defects in the cranial region, similar to those found in the LMO4 knockout mice (McMahon *et al*., 1998). Similarly, Smad5 knockout mice exhibit

failure of cranial neural tube closure and exencephaly (Chang *et al*., 1999). Furthermore, mice deleted for the c-ski gene, which encodes a transcriptional repressor involved in TGFβ/BMP signaling, show both exencephaly and defects in the basal skull bones similar to those found in LMO4 knockout mice (Berk *et al*., 1997). Thus, it is possible that altered signaling by TGFβ superfamily ligands plays roles in some of the abnormalities in LMO4 knockout mice.

In addition to a developmental role, there are several lines of evidence suggesting that LMO4, like other members of this gene family, may play roles in oncogenesis. LMO4 was originally identified as an autoantigen in human breast cancer (Racevskis *et al*., 1999) and subsequently shown to be upregulated in over 50% of breast cancer cases (Visvader *et al*., 2001). Additionally, it was found that LMO4 could interact with the BRCA1 tumor suppressor gene (Sum *et al*., 2002). Consistent with a role in mammary epithelial cells, we have shown that overexpression of a dominant-negative LMO4 inhibits ductular and lobuloalveolar development in the mammary glands of transgenic mice (Wang *et al*., 2004), and others have demonstrated that mammary gland-specific deletion of the LMO4 gene leads to impaired lobuloalveolar development during pregnancy (Sum *et al*., 2005c). LMO4 has also been shown to be upregulated at the invasive fronts of oral cancers, suggesting a role in cancer cell invasion (Mizunuma *et al*., 2003). In the prostate, LMO4 was downregulated during tumor progression and lowered in hormone refractory tumors (Mousses *et al*., 2002). In breast cancers and in breast cancer cell lines, LMO4 levels appear to be disproportionately upregulated as compared to the levels of Clim factors (Visvader *et al*., 1997; Wang *et al*., 2004). Therefore, the effects we have observed may have particular relevance for such situations where LMO4 and Clim levels are not coordinately regulated.

Materials and methods

Cell culture, retroviruses, and transfection assays

Normal HMEC were purchased and cultured according to protocols from Cambrex. The murine mammary epithelial (NMuMG) cells, human embryonic kidney (HEK293T) cells, and human breast cancer cell line T47D were cultured according to the ATCC protocol.

Retroviruses expressing LMO4 gene and control protein were based on the Retro-X™ System from BD Biosciences. Construction of the LMO4 retroviruses and the infection of virus into cells were performed according to the manufacturer's protocol. LMO4 was fused in frame at the C-terminus to the tandem affinity purification (TAPc) tag, which contains two IgG-binding domains of *Staphylococcus aureus* protein A and a calmodulin-binding peptide separated by a TEV protease cleavage site (Puig *et al*., 2001). Another vector was created in which LMO4 was fused in frame at the C-terminal site to GFP. Retrovirus was harvested from the stably transfected packaging cell line GP2-293, and the titer of virus was determined using NIH3T3 cell. In experiments, cells were infected with equivalent virus titer for each construct and for the same length of time. Protein expression was determined by Western blotting to ensure similar expression from the control and experimental viruses.

Transient transfections and luciferase reporter assays were performed as previously described, using calcium precipitation for HEK293 cells, and Lipofectamine™ 2000 (Invitrogen) for HME and NMuMg cells (Sugihara *et al*., 2001). Luciferase activity was normalized for differences in transfection efficiency, using the Renilla luciferase vector (Promega). The plasmids used in these studies have been previously described: 9xGAGA-Luciferase (Dennler *et al*., 1998), pCS2-MT-LMO4 (Sugihara *et al*., 1998), and pCMV5-TβR1-AAD (Chen *et al*., 1997).

The LMO4-specific siRNAs, which were designed based on the human LMO4 mRNA sequence (accession number, NM_006769), were obtained from Ambion. The target sequences of the LMO4 duplex siRNAs are: GGCAATGTGT ATCATCTTA (LMO4#1), GGTCTGCTAAAAGGTCAGA (LMO4#2), and GGAAACGTGTTTCAATCAA (LMO4#3). The control siRNA was unrelated to the LMO4 sequence and not known to affect any endogenous genes (Ambion). The siRNAs were introduced into T47D cells using RNAiFect transfection reagent (Qiagen). For transcriptional assays, LMO4 shRNAs and the control shRNA were synthesized and cloned into RNAi-Ready PSIREN-RetroQ-ZsGreen vector (BD Biosciences). The duplex sequences of the LMO4 shRNAs are: 5′-gatccggcaatgtgtatcatcttattcaagagataagatgatac acattgccttttg-3′ (shRNA #1), 5′-gatccggaaacgtgtttcaatcaattcaa gagattgattgaaacacgtttccttttg-3′ (shRNA #3), and 5′-gatccgtgcg ttgctagtaccaacttcaagagatttttttacgcgtg-3′ (shRNA control).

Recombinant mature human TGFβ1 (R&D Systems) was used according to the manufacturer's recommendations. Unless otherwise indicated, all other chemicals were from Fisher/ICN.

Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and complementary DNA was synthesized using 5 μg of total RNA with the High-Capacity cDNA archive kit (Applied Biosystems) (Lin *et al*., 2004). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7900HT platform (384-well plates; Applied Biosystems), following standard protocols from the supplier to detect threshold cycle (C_t). ΔC_t values were calculated by comparing the C_t measurements of experimental wells to the untreated (basal) wells that were infected with the control virus. All values were then normalized to 18S rRNA to obtain $\Delta\Delta C_t$ values.

Co-immunoprecipitations, Western blots, and GST pull-down assays

Co-immunoprecipitations of extracts from transfected HEK293T cells were performed as previously described (Sugihara *et al*., 2001), using MT (myc) antibody (Invitrogen; R950-25) recognizing tagged LMO4, and HA antibody (Covance; MMS-101R) detecting tagged Smads. The following vectors, pCMV5/Smad1-HA, pCMV5/Smad2-HA, pCMV5/Smad4-HA, and pGCN/HA-Smad5, were described previously (Chen *et al*., 1997; Hata *et al*., 1997). For co-immunoprecipitation of endogenous LMO4 and Smad2 proteins in HEK293T cells, we used antibodies directed against LMO4 (Santa Cruz; SC-11122) and Smad2 (Zymed; 51-1300). For GST pull-down assays, Smad mutant genes were generated by PCR-based deletion, followed by cloning into vectors allowing *in vitro* transcription/translation; the sequences were confirmed by DNA sequencing. Western blot analysis was performed as described previously (Wang *et al*., 2004), using antibodies to

phosphor-Smad2 (Cell Signaling; 3101), LMO4 (Sum *et al*., 2002), MT (Invitrogen; R950-25), HA (Covance; MMS-101R), GFP (Upstate Cell Signaling Solution; 06–896), TAPc (Peroxidase-anti-peroxidase (PAP) antibody, Sigma-Aldrich; P-2026), GAPDH (Ambion; 4300), and β-actin (Santa Cruz; SC-8432).

The GST pull-down assays were performed as previously described (de la Calle-Mustienes *et al*., 2003). Briefly, GST protein or GST-LMO4 fusion protein were incubated with ^{35}S-labeled *in vitro* translated Smad proteins at room temperature for 30 min. After washing three times, the glutathione–agarose beads were resuspended in SDS sample buffer, boiled, and analysed on 10% SDS–polyacrylamide gels.

ChIP

ChIP assays were performed according to the protocol from Upstate Cell Solution. Chromatinized DNA was crosslinked in 1% formaldehyde for 10 min at 37°C. Cells were then washed twice using ice-cold phosphate-buffered saline containing protease inhibitors (Roche Applied Science; 10752800) and then harvested in PBS with protease inhibitors. Thereafter, cells (1×10^6) were resuspended in 0.2-ml SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated on ice for 10 min, and sonicated to reduce the chromatin DNA length to 1 kb. The lysates were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and precleared with sperm DNA-protein A-agarose beads (Upstate Cell Signaling Solutions) at 4°C for 1 h. Following overnight incubation with 2 μg of anti-MT or IgG, immune complexes were immobilized by salmon sperm DNA protein A agarose beads. After extensive washing and elution with 1% SDS and 0.1 M $NaHCO_3$, crosslinks were reversed by incubation at 65°C for 4 h in the presence of 0.2 M NaCl. The released DNA was phenol–chloroform-purified, and the PAI-1 and GAPDH promoter sequences were detected by PCR followed by agarose gel visualization. The ChIP primers for PAI-1 are 5′-CCT CCAACCTCAGCCAGACAAG-3′ (forward) and 5′-CCCAG CCCAACAGCCACAG-3′ (reverse) (Kurisaki *et al*., 2003). The primers for GAPDH are 5′-CGGCTACTAGCGGTTTT ACG-3′ (forward) and 5′-AAGAAGATGCGGCTGACTGT-3′ (reverse).

Cell growth assays

Cells were incubated overnight at a density of 5000 cells/well in 96-well plates, and treated with TGFβ1 (1 ng/ml) for 24 h. Then, cells were grown in a fresh growth medium for up to 5 days. Cell growth was assessed daily using the conversion of MTT to formazan production (Matsuda *et al*., 2002). Briefly, cells from 10 wells were incubated with MTT (62.5 μg/well) for 4 h. Cellular MTT was solubilized with acidic isopropanol, and absorbance was measured at 570 nm with an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). Results were plotted as the mean ± s.d. of 10 determinations for each time point. Four independent experiments were performed; the data from a representative experiment are shown.

Cell proliferation assays

To determine cell proliferation, cells were labeled with 5-(and 6-) carboxy fluoroscein diacetate succimidyl ester (CFSE; Molecular probes, Eugene, USA) to quantify cell division (Lee *et al*., 2004). Briefly, cells were resuspended in PBS at 2×10^7 cells/ml and labeled by incubation in 5 μM CFSE for 8 min at RT. Cells were then quenched with Fetal Bovine Serum, washed three times with PBS and plated onto six-well plates (10 000 cells/well). On the second day, cells were treated with TGFβ1 (1 ng/ml) for 4, 8, 16, 24 h, and then grown in fresh medium for another 3 days. Cells were detached by 0.05% trypsin (Invitrogen), suspended in 1 ml PBS, and analysed by a FACSCaliber flow meter (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

Apoptosis assays

For annexin V staining, cells were seeded at a density of 1×10^5 cells/60-mm dish on day 0. On day 1, cells were treated with TGFβ1 (1 ng/ml) for 24 h. Then, cells and supernatant were collected and stained with annexin-V-FITC and propidium iodide (PI), using the Annexin V-FLUOS Staining Kit (Roche Applied Science; 1858777). Duplicate samples were analysed on a FACSCaliber flow meter (Becton Dickinson, Mountain View, CA, USA) using CellQuest software.

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The Grainyhead-like epithelial transactivator Get-1/Grhl3 regulates epidermal terminal differentiation and interacts functionally with LMO4

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Abstract

Defective permeability barrier is an important feature of many skin diseases and causes mortality in premature infants. To investigate the control of barrier formation, we characterized the epidermally expressed Grainyhead-like epithelial transactivator (Get-1)/Grhl3, a conserved mammalian homologue of Grainyhead, which plays important roles in cuticle development in *Drosophila*. Get-1 interacts with the LIM-only protein LMO4, which is co-expressed in the developing mammalian epidermis. The epidermis of *Get-1*$^{-/-}$ mice showed a severe barrier function defect associated with impaired differentiation of the epidermis, including defects of the stratum corneum, extracellular lipid composition and cell adhesion in the granular layer. The *Get-1* mutation affects multiple genes linked to terminal differentiation and barrier function, including most genes of the epidermal differentiation complex. *Get-1* therefore directly or indirectly regulates a broad array of epidermal differentiation genes encoding structural proteins, lipid metabolizing enzymes and cell adhesion molecules. Although deletion of the *LMO4* gene had no overt consequences for epidermal development, the epidermal terminal differentiation defect in mice deleted for both *Get-1* and *LMO4* is much more severe than in *Get-1*$^{-/-}$ mice with striking impairment of stratum corneum formation. These findings indicate that the *Get-1* and *LMO4* genes interact functionally to regulate epidermal terminal differentiation.

Keywords: Get-1; LMO4; Epidermal differentiation; Epidermal differentiation complex; Epidermal barrier; Grainyhead

Introduction

The stratified mammalian epidermis develops from somatic ectoderm late in embryogenesis. As keratinocytes move from the proliferating basal cell layer towards the surface, they undergo a series of differentiation steps to form morphologically distinct suprabasal layers: the spinous, granular and cornified layers. One of the key roles of this process is to form an effective permeability barrier, which depends on several components of the cornified and granular layer (Candi et al., 2005). As a consequence of defective epidermal differentiation, premature infants suffer both from increased transepidermal water loss and percutaneous absorption of chemicals, as well as a fragile skin (Shwayder and Akland, 2005). Barrier function is also defective in several hereditary and acquired inflammatory skin diseases (Nickoloff, 2006).

The cornified envelope is composed of a complex of cross-linked structural proteins that form a rigid structure in the dead cells of the cornified layer. These cells are surrounded by lipid lamellae composed of a mixture of lipids, some of which are cross-linked to the cornified envelope. Cross-linking enzymes such as transglutaminases and metabolic enzymes expressed in the granular layer are responsible for protein cross-linking and

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lipid synthesis. In addition, cell–cell adhesion molecules in the granular layer, including desmosomes and tight junction proteins, are critical for an effective barrier (Segre, 2003).

Very little is known about the coordinated regulation of gene expression that is required for formation of the epidermal barrier (Segre, 2003). In an effort to understand transcriptional regulation of epidermal differentiation, we previously identified two interacting proteins in the developing epidermis: the LIM-only factor LMO4 and Grainyhead-like epithelial transactivator (Get-1; also referred to as Grainyhead-like transcription factor 3/Grhl3) (Kudryavtseva et al., 2003; Sugihara et al., 1998). LMO4 is a transcriptional co-regulator thought to coordinate larger transcriptional complexes by interacting with DNA-binding proteins (Sugihara et al., 1998). Get-1 is a conserved mammalian homologue of the DNA-binding protein Grainyhead (Kudryavtseva et al., 2003; Ting et al., 2003b), which plays an important role in *Drosophila* cuticle formation (Bray and Kafatos, 1991; Mace et al., 2005). The conserved expression of *Drosophila* Grainyhead and Get-1 suggested a role for Get-1 in mammalian epidermis formation (Kudryavtseva et al., 2003).

During embryonic development of the epidermis, Get-1 and LMO4 are co-expressed in the epidermal keratinocytes and interact *in vitro* (Kudryavtseva et al., 2003), suggesting that both Get-1 and LMO4 might be important for epidermal differentiation. To test this prediction, we studied epidermal development in mice deleted for *Get-1* and *LMO4*. *Get-1* deletion disrupted terminal differentiation and barrier function of the epidermis. Ting et al. (2005) suggested that down-regulation of transglutaminase 1 (Tgm1), a key cross-linking enzyme in epidermal differentiation, is responsible for the epidermal barrier defect and impaired wound healing in *Get-1*$^{-/-}$ mice. In contrast, we found that *Get-1* regulates multiple components of the epidermal barrier in addition to Tgm1, including structural and cell adhesion genes, as well as the lipid component. Furthermore, knockout of *LMO4* enhanced the terminal differentiation defect in *Get-1*$^{-/-}$ mice, indicating functional interactions between *Get-1* and *LMO4* in the regulation of the terminal differentiation program in the epidermis.

Materials and methods

Generation of Get-1$^{-/-}$ *and LMO4*$^{-/-}$ *mice*

A gene-targeting vector was constructed using PCR from 129/SvJae mouse genomic DNA. A 5′ homology arm (3602 bp), a 3′ homology arm (2891 bp) and a loxP arm (2257 bp) were isolated and cloned into the pFOz (3L) vector. The linearized targeting vector was introduced into ES cells by electroporation. Electroporated C57Bl/6J ES cells were grown on selection medium containing G418 (GIBCO BRL). G418-resistant ES cells were isolated and screened by Southern blotting of *Kpn*I-digested genomic DNA with 5′ and 3′ probes, and positive ES cell clones were used to generate the *Get-1* chimeric mice. The chimeric mice were intercrossed with C57Bl/6J mice to obtain *Get-1* floxed mice. The *Get-1* heterozygous knockout mice were obtained by crossing the floxed mice with a Cre-deletor (Schwenk et al., 1995) to remove the DNA fragment from exon 4 to exon 7. The generation of *LMO4*$^{-/-}$ mice was previously described (Lee et al., 2005). Sequences of oligonucleotides used for genotyping and RT-PCR of Get-1 mRNA are provided in Supplementary Table 1.

RT-PCR analysis

Semi-quantitative RT-PCR was performed on total RNA prepared from E18.5 backskin of WT and *Get-1*$^{-/-}$ embryos using Trizol Reagent (Invitrogen) and the High Capacity cDNA Archive Kit (Applied Biosystems). Reactions were sampled after 25, 28 and 30 cycles at different PCR conditions to monitor product accumulation. Sequence of primers is in Supplementary Table 1.

Histology, immunohistology and BrdU staining

Backskin was fixed in 10% formalin, paraffin-embedded and 6-μm sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry, skin tissues were fixed in 6 parts 100% Ethanol, 3 parts water and 1 part Formaldehyde. Antigen-retrieval was performed by heating slides to 95°C for 10 min in 0.01 M citrate buffer (pH 6) in a microwave oven. The sections were then immunostained by the ABC peroxidase method (vector) with diaminobenzidine as the enzyme substrate and hematoxylin as a counterstain. For immunofluorescence, 6-μm-thick fresh frozen sections were air-dried and then washed with PBS. Later, these sections were soaked in blocking solution for 30 min, incubated with occludin mAb for 2 h, washed three times with blocking solution, then incubated with FITC anti-rat IgG pAb (Oncogene Research Products) and counterstained with DAPI in mounting media. For BrdU detection, BrdU (0.05 mg/g) was injected intraperitoneally 2 h prior to sacrifice of the pregnant mother. Embryos were fixed in 10% buffered formalin and paraffin embedded. Slides were pre-treated in 1 M HCl for 1 h at 37°C prior to adding antibody. The primary antibodies used in immunostaining were as follows: rabbit anti-mouse MK5 (Covance), mouse anti-human cytokeratin 10 (DakoCytomation), rabbit anti-mouse filaggrin (Covance), rabbit anti-mouse loricrin (Covance), mouse anti-mouse occludin (Zymed), rabbit anti-mouse claudin 1 (Abcam), rabbit anti-mouse claudin 4 (Abcam), rabbit anti-mouse caspase 14 (Abcam), Rabbit anti-mouse involucrin (Covance) and anti-bromodeoxyuridine (Roche).

In situ hybridization

In situ hybridization studies with ^{35}S-labeled cRNA probes were performed on frozen sections counterstained with bisbenzamide as described previously (Andersen et al., 1995).

Epidermal barrier permeability assay

To assess the epidermal permeability barrier, we used the skin permeability assay described previously (Hardman et al., 1998). After staining, the embryos were photographed using a Nikon E995 digital camera.

Transmission electron microscopy

Fresh skin was dissected into small pieces and fixed by immersion in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4). Tissues were fixed in room temperature and then washed for four times with PBS. They were postfixed with 0.2% ruthenium tetroxide (RuO_4) and dehydrated through graded ethanol series and embedded in agar 100 resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined on a transmission electron microscope.

Preparation of cornified envelopes (CEs) and sonication experiments

Embryo skin was processed with dispase at 37°C for 30 min and epidermis was isolated. CEs were prepared by heating epidermis at 95°C for 10 min in preheated extraction buffer (0.1 M Tris pH 8.5, 2% SDS, 20 mM DTT, 5 mM EDTA pH 7.5). CEs were collected by centrifugation of 5000×*g* for 15 min and stored in 500 μl extraction buffer. For sonication, CEs were diluted in extraction buffer and the CE suspension was sonicated in Eppendorf tubes on ice for different time points. CE aliquots were photographed.

Lipid analysis

Epidermis was isolated from e18.5 WT, heterozygous and Get-1 knockout embryos as described above. Lipid analysis from pooled epidermis preparations was performed as previously described (Law et al., 1995). Similar results were obtained in four independent experiments.

RNA isolation and microarray experiments

The same region of the mouse backskin was excised from three *Get1-1*$^{+/+}$ and three *Get1-1*$^{-/-}$ mice at e18.5. Microarray experiments were performed as previously described except we used Affymetrix Murine Genome 430 2.0 arrays (45,037 probe sets) and washed according to manufacturer's recommendations (Affymetrix, Santa Clara, CA) (Lin et al., 2004).

Cyber-T analysis

After excluding absent genes, the raw expression values from replicate samples were analyzed by the Cyber-T program (Baldi and Long, 2001), which identifies statistically significant differentially expressed genes. We implemented the following filtering criterion to exclude absent genes from subsequent analysis: all three replicate samples of either Get1-1$^{+/+}$ or Get1-1$^{-/-}$ mice must have "present" or "marginally present" calls, as determined by MAS 5.0. To determine the global false positive rate inherent in multiple hypotheses testing

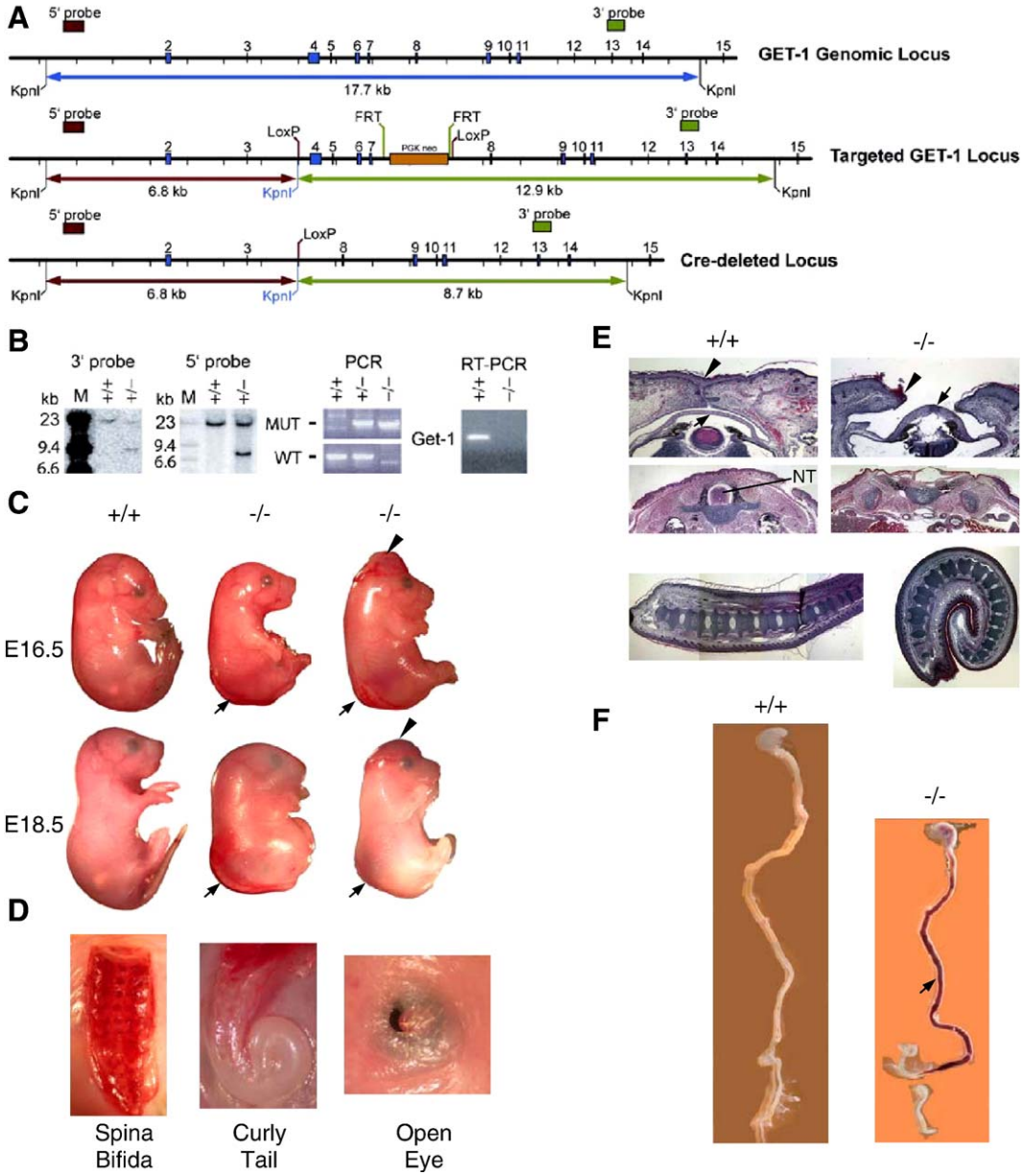


Fig. 1. Generation and phenotypes of *Get-1*$^{-/-}$ mice. (A) Strategy for targeted deletion of the *Get-1* gene. Exons are represented as boxes. Locations of probes for Southern blots are indicated. (B) The first two panels show Southern blot analysis of *Kpn*I-digested genomic DNA from WT (+/+) and targeted (+/−) embryonic cells with 3′ and 5′ probes. The third panel shows PCR identification of genotypes of mice using primers detecting normal (WT) and deleted (MUT) *Get-1* loci. The fourth panel shows absence of Get-1 mRNA in *Get-1*$^{-/-}$ mice by RT-PCR. (C) Appearance of *Get-1*$^{-/-}$ mice at e16.5 and e18.5. Arrowheads indicate exencephaly and arrows indicate spina bifida. (D) Higher magnification images of spina bifida, curly tail and open-eye phenotypes in *Get-1*$^{-/-}$ mice. (E) Histology of e18.5 embryos shows open-eye, spina bifida and curly tail phenotypes. Arrowheads point to the eyelid closure front and arrows point to the cornea. (F) Overview of the intestine of *Get-1*$^{-/-}$ mice. Arrow points to blood in intestine.

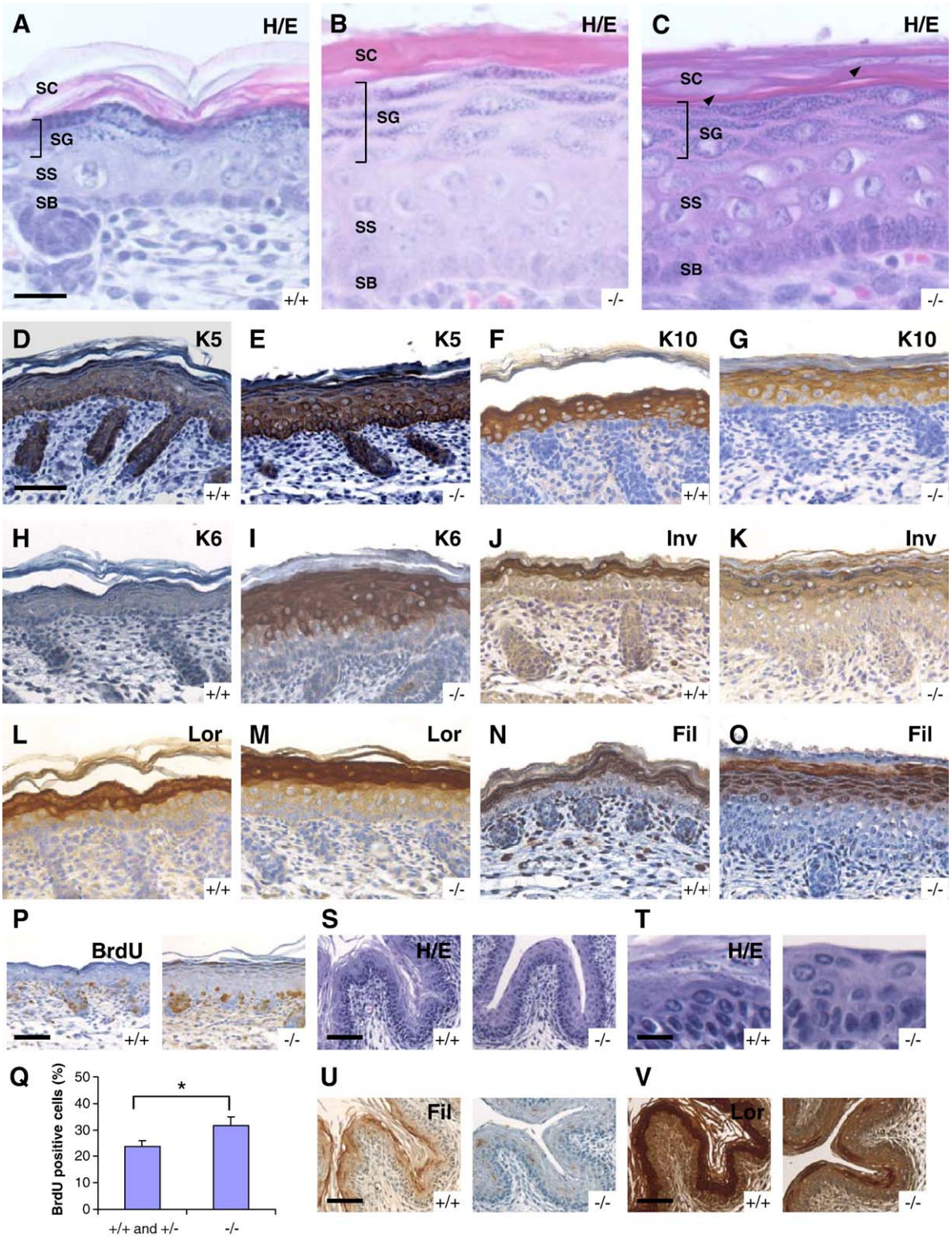


Fig. 2. Impaired epithelial differentiation in *Get-1*$^{-/-}$ epidermis and forestomach. (A–C) Histological analysis of e18.5 backskin from WT (A) and *Get-1*$^{-/-}$ (B–C) embryos. Arrowheads in C point to nuclei in the stratum corneum. (D–O) Immunostaining analysis of e18.5 backskin from WT (D, F, H, J, L and N) and *Get-1*$^{-/-}$ (E, G, I, K, M and O) embryos. Antibodies to the following keratinocyte differentiation markers were used, K5 (D–E), K10 (F–G), K6 (H–I), involucrin (J–K), loricrin (L–M) and filaggrin (N–O). (P) BrdU staining of e18.5 backskin from WT (left panel) and *Get-1*$^{-/-}$ (right panel) embryos. (Q) The ratio of BrdU positive cells in the basal cell layer of e18.5 backskin with the indicated genotypes. Results represent mean and standard error. The asterisk denotes statistically significant difference between WT/heterozygous and *Get 1*$^{-/-}$ mice ($P < 0.02$). (S) Histological analysis of forestomach epithelium from e18.5 mice with the indicated genotypes. (T) Higher magnification of the images shown in S. (U) Filaggrin immunostaining of forestomach epithelium from e18.5 mice with the indicated genotypes. (V) Loricrin immunostaining of forestomach epithelium from e18.5 mice with the indicated genotypes. Scale bars: A–C, 25 μm; D–S, U and V, 50 μm; T, 12.5 μm. H/E, hematoxylin/eosin staining; SB, basal layer; SC, cornified layer; SG, granular layer; SS, spinous layer.

of high-dimensional DNA array data, the posterior probability of differential expression (PPDE) was calculated using the *P* values of log-transformed data. The PPDE for the selected cut-off *P* value of 0.0025 is 0.80, which indicates that the false discovery rate is within 20%. Overrepresented gene ontology biological process categories and chromosomal band localization for differentially expressed genes were determined using the DAVID (Database for Annotation, Visualization and Integrated Discovery) 2.0 program (Dennis et al., 2003).

Batch extraction and analysis of cis-regulatory regions (BEARR)

BEARR was used to determine the possible Get-1 binding sequence using position weight matrix score (PWM score ≥6.73) by extracting the promoter regions (2 kb upstream region of transcription start site) of significantly altered genes in the microarray (Vega et al., 2004). The PWM input used for the analysis was from the core Get-1 DNA binding consensus sequence (AACCGGTT) derived from a previously published CASTing results (Ting et al., 2005). The sequence and annotation database selected for BEARR analysis was the Mouse Genome Assembly NCBI Build 33. Of the 162 unique genes identified by Cyber-T to be downregulated, BEARR was able to obtain 2 kb upstream sequence for 136 genes, and 35 of these genes (25.7%) have sites with PWM scores ≥6.73. Of the 69 unique genes identified by Cyber-T to be upregulated, BEARR was able to obtain 2 kb upstream sequence for 59 genes, and 12 of these genes (20.3%) have sites with PWM scores ≥6.73. To estimate the expected number of genes identified to have upstream sites with PWM scores ≥6.73 by chance (false positives), we systematically performed PWM analysis on statistically significant non-regulated genes by Get-1 ($P>0.99$ from Cyber-T analysis; 246 probe sets, corresponding to 192 unique genes). For these non-regulated genes, BEARR was able to obtain 2 kb upstream sequence for 173 genes, and 31 of these genes (17.9%) have sites with PWM scores ≥6.73. In running the ConSite program (Sandelin et al., 2004), we used 75% as the cut-off threshold for conservation of Get-1 binding sites and genomic regions with a sliding window of 50 bp.

Electrophoretic mobility shift assay (EMSA)

Sequences extracted by BEARR were used as unlabeled competitor oligonucleotides in the competition EMSA (Supplementary Table 2). Get-1 protein (0.2 ng/ml) was incubated with [γ^{32}]P-labeled duplex Get-1 consensus sequence (TCCTGTTAAACCGGTTTTTCTAGT) and EMSA performed as previously described (Kudryavtseva et al., 2003). The DNA-protein complexes in the various bands were quantified by cutting them out and measuring their counts using a scintillation counter. Data were expressed as percent binding relative to that determined in the absence of a competitor. A competitive binding curve was used to approximate the concentration of the unlabelled oligonucleotides (IC_{50}) required for 50% inhibition of binding. The computed relative affinity is the IC_{50} of the gene relative to the IC_{50} of Tgm1.

Co-immunoprecipitations and Western blots

Expression plasmids, pCS2-MT-LMO4 and pCDNA-HA-Get-1 were transiently transfected into HEK293T cells and cell extracts prepared as previously described (Lu et al., 2006). Cell extracts were precipitated with an HA antibody (Covance; MMS-101R) and Western blots were performed with MT antibody (Upstate; 06-340). Input extracts were analyzed by Western blots with the same antibodies.

Results

Get-1 regulates terminal differentiation of the epidermis and the forestomach epithelium

To test whether Get-1 and LMO4 play roles in epidermal development, we studied mice deleted for *Get-1* and *LMO4*. We first generated *Get-1*$^{-/-}$ mice lacking exons 4 to 7, which encode for the activation domain and part of the DNA-binding

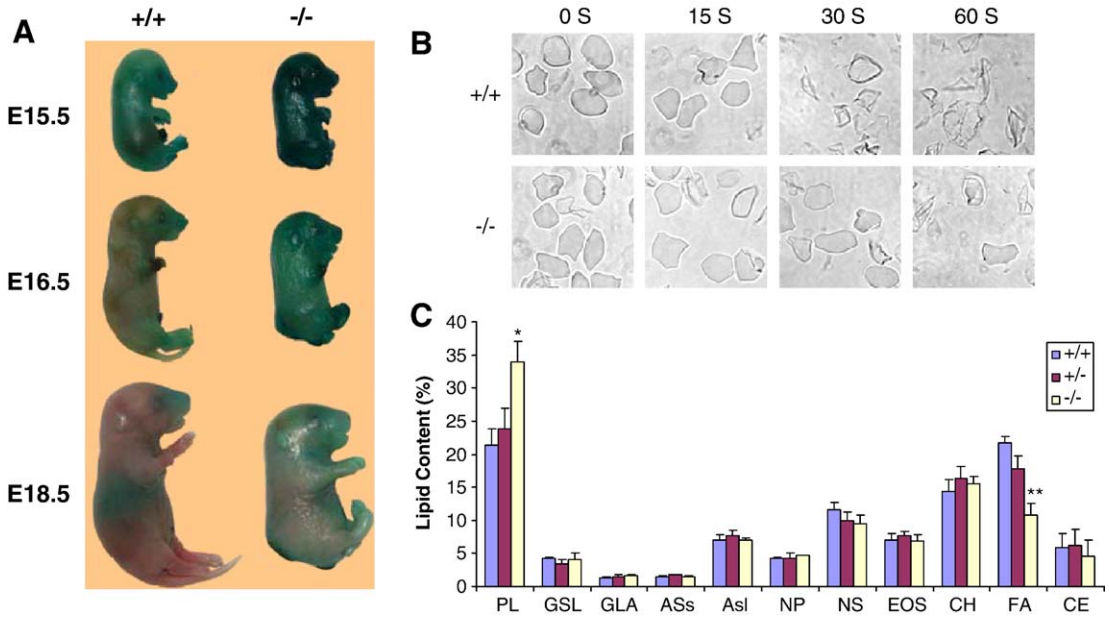


Fig. 3. Defective epidermal permeability barrier and abnormal epidermal lipid composition in *Get-1*$^{-/-}$ mice. (A) Epidermal permeability barrier assay by X-gal staining of mouse embryos of the indicated ages and genotypes. (B) Ultrasound fragility analysis of cornified envelopes isolated from the epidermis of e18.5 mice of the indicated genotypes. Shown are images of cornified envelopes after ultrasound treatment for 0, 15, 30 and 60 s. (C) Lipid composition of epidermis of mice with the indicated genotypes. Shown are means and standard errors from pooled epidermal samples in four different experiments. * and ** denote $P<0.01$ and $P<0.002$, comparing WT and *Get 1*$^{-/-}$ epidermis. PL, phospholipids; GSL, glucosylceramides; GLA, acylglucosylceramide; ASs, ceramide containing sphingosine and short α-hydroxyacids; Asl, ceramide containing sphingosine and long α-hydroxyacids; NP, ceramide containing sphingosine and short α-hydroxyacids; NS, ceramide containing sphingosine and normal fatty acids; EOS, acylceramide consisting of omega-hydroxyacids amide-linked to sphingosine and bearing linoleate ester-linked to the omega-hydroxyl group; CH, cholesterol; FA, fatty acid; SE, cholesterol ester.

domain (Figs. 1A–B). The *Get-1*$^{-/-}$ mice died at birth with neural tube closure defects characterized by spina bifida (100%) and exencephaly (14%), and exhibited tail abnormalities (100%), primarily curly tail (Figs. 1C–E and Supplementary Table 3). These phenotypes are similar to those previously described in mice deleted for part of exon 2 and exon 3 of the *Get-1* gene (Ting et al., 2003a). However, in the present study, the incidence of exencephaly was 7 times higher and additional abnormalities were observed, including an open-eye phenotype (7%; Supplementary Table 3) and shorter intestine with blood in the lumen (Fig. 1F). The mean intestine lengths of WT and *Get 1*$^{-/-}$ mice were 6.8 and 5.9 cm, respectively ($P<0.002$). The eye and gut phenotypes expand the known functions of Get-1.

To understand the role of *Get-1* in epidermal development, we examined skin histology and marker expression in *Get-1*$^{-/-}$ mice from embryonic days (e) 14.5 to 18.5 (Figs. 2A–O and Supplementary Fig. 1). At e14.5, the skin histology was normal but by e16.5 there was thickening of the epidermis, which became more pronounced at e18.5 (Figs. 2A–C and Supplementary Fig. 1). At this stage, the cornified layer was more compact and often contained nuclei (Fig. 2C). The granular layer was thicker due to an increased number of cell layers and the cells of the most superficial layers being more cuboidal than normal. The spinous layer was also thicker and the basal layer appeared disorganized. Keratin 5 and 10 expression appeared normal whereas keratin 6 expression, often associated with increased epidermal proliferation, was strongly upregulated in *Get-1*$^{-/-}$ epidermis, especially close to the spina bifida (Figs. 2D–I). Examination of the terminal differentiation markers involucrin, loricrin and filaggrin revealed that the onset of loricrin expression was delayed at

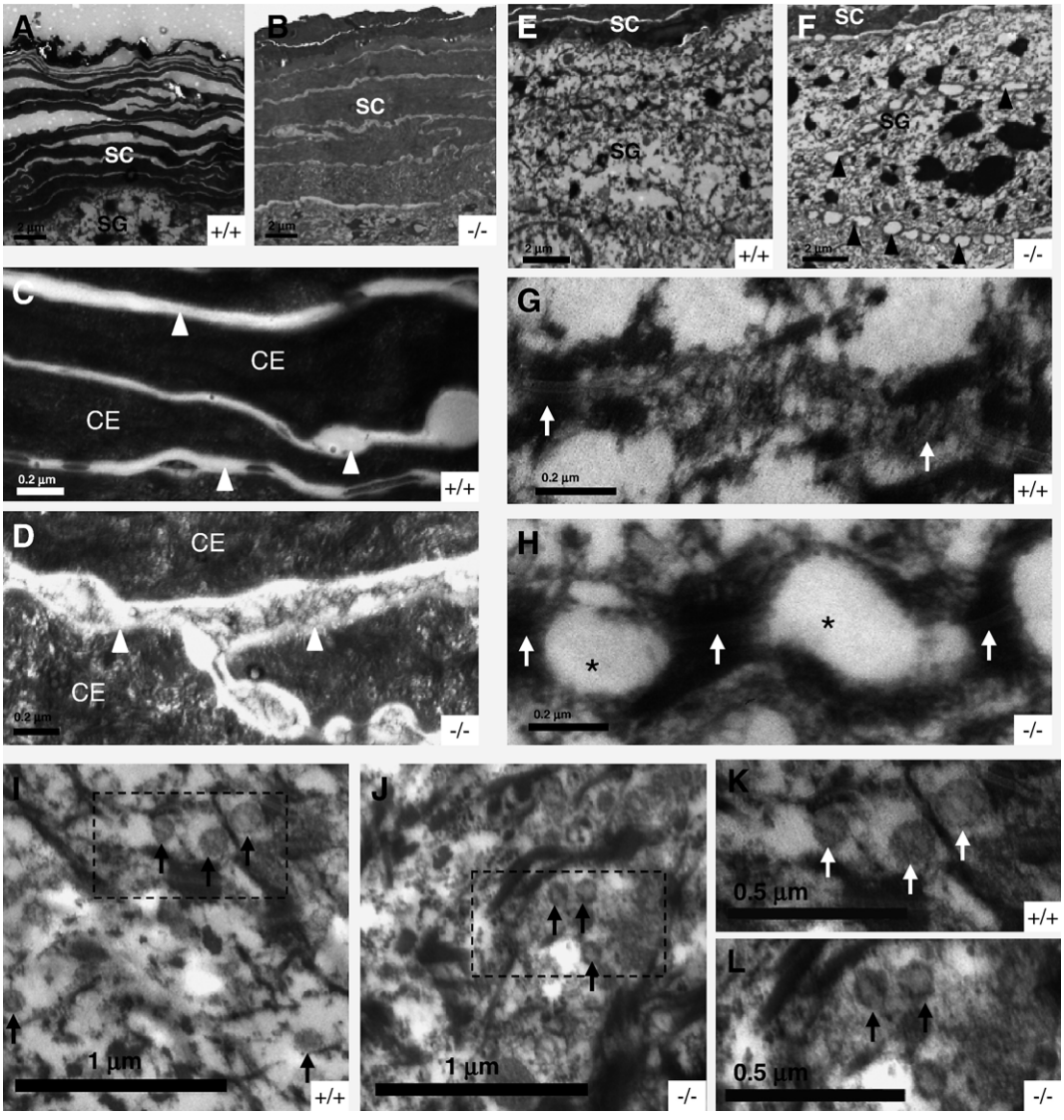


Fig. 4. Ultrastructure of *Get-1*$^{-/-}$ epidermis. (A–B) Stratum corneum of WT (A) and *Get-1*$^{-/-}$ (B) mice at e18.5. (C–D) Higher magnification of WT (C) and *Get-1*$^{-/-}$ (D) stratum corneum. Arrowheads indicate intercellular spaces. (E–F) Granular layer of WT (E) and *Get-1*$^{-/-}$ (F) epidermis from e18.5 embryos. Arrowheads indicate separated cell–cell adhesions in *Get-1*$^{-/-}$ the epidermis. (G–H) Higher magnification of the granular layer of WT (G) and *Get-1*$^{-/-}$ (H) mice. Arrows indicate desmosomes. Asterisks indicate the abnormally separated intercellular spaces. (I–L) Lamellar bodies indicated with arrows in the granular layer of e18.5 epidermis from WT (I–K) and *Get-1*$^{-/-}$ (J–L) embryos. CE, cornified envelope; SC, stratum corneum; SG, granular layer.

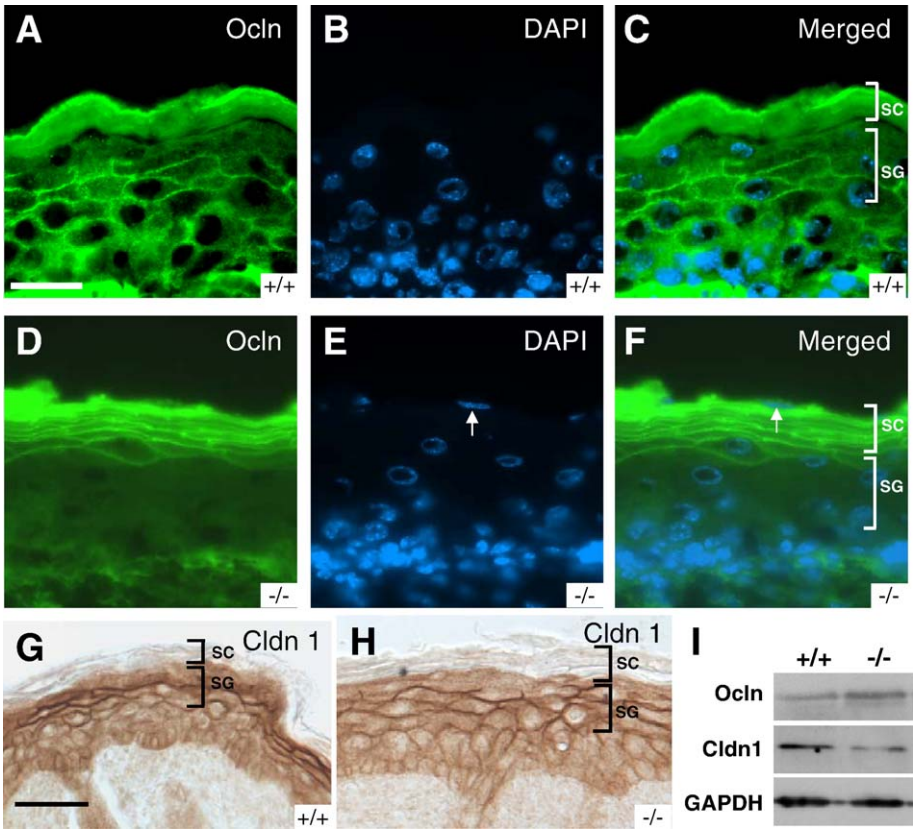


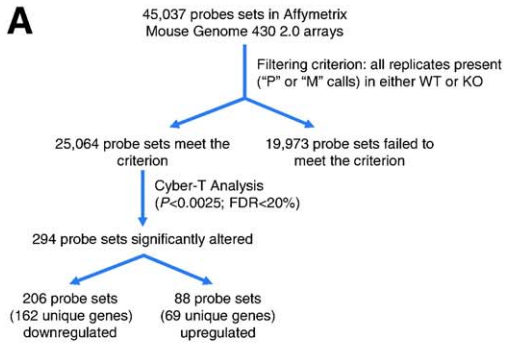
Fig. 5. Expression pattern of occludin and claudin 1 in WT and *Get 1*$^{-/-}$ epidermis. (A) Immunofluorescence analysis of occludin expression in WT epidermis. (B) DAPI staining showing nuclei in the same section as panel A. (C) The merged image of panels A and B. (D) Immunofluorescence analysis of occludin expression in *Get 1*$^{-/-}$ epidermis. (E) DAPI staining showing nuclei in the same section as panel D. (F) The merged picture of panels D and E. (G–H) Immunohistochemistry analysis of claudin 1 expression in WT (G) and *Get 1*$^{-/-}$ (H) epidermis. SC, cornified layer; SG, granular layer. A nucleus in cornified layer is indicated by arrow. (I) Western blot analysis of occludin and claudin 1 in WT and *Get 1*$^{-/-}$ epidermis. Ocln, occludin; Cldn1, claudin 1. Scale bars: panels A–F, 25 μm; G–H, 50 μm.

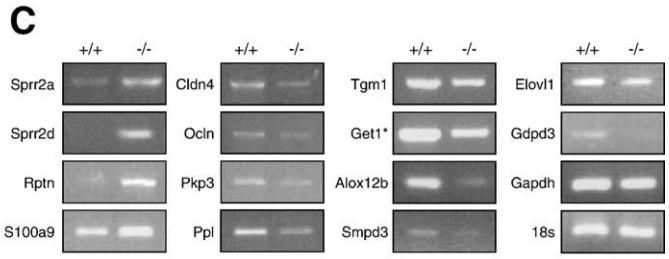
e14.5 whereas the other two terminal differentiation markers were expressed at the appropriate time (Supplementary Fig. 1). However, all three markers showed an expanded domain of expression at e18.5, corresponding to expansion of the granular layer (Figs. 2J–O). Involucrin expression was decreased slightly at e16.5 (Supplementary Fig. 1) and clearly at e18.5 (Figs. 2J–K). Together, these abnormalities demonstrate altered terminal differentiation of the *Get-1*$^{-/-}$ epidermis. Consistent with epidermal hyperplasia, there was a mild, yet statistically significant increase in keratinocyte proliferation in the basal cell layer of the *Get-1*$^{-/-}$ epidermis (Figs. 2P–Q). The forestomach epithelium, which is similar to the epidermis, showed reduced number of granules in the superficial layers (Figs. 2S–T) and decreased expression of filaggrin and loricrin (Figs. 2U–V), signifying impaired terminal differentiation of the forestomach epithelium. These experiments demonstrate that *Get-1* plays important roles in the differentiation of stratified epithelia of both ectodermal and endodermal origin, thus expanding the role for this mammalian homologue of *Drosophila* Grainyhead.

Get-1 regulates the lipid composition, cell–cell adhesion and barrier function of the epidermis

One key role of the epidermis is the formation of an effective permeability barrier. By performing an *in situ* permeability assay (Hardman et al., 1998), impaired barrier formation was evident in *Get-1*$^{-/-}$ mice at e16.5 and e18.5 (Fig. 3A). An effective barrier depends in part on the structural integrity of the cornified envelope. To test the fragility of cornified envelopes, we exposed them to ultrasound for different time periods. Interestingly, the cornified envelope appeared normal or slightly less

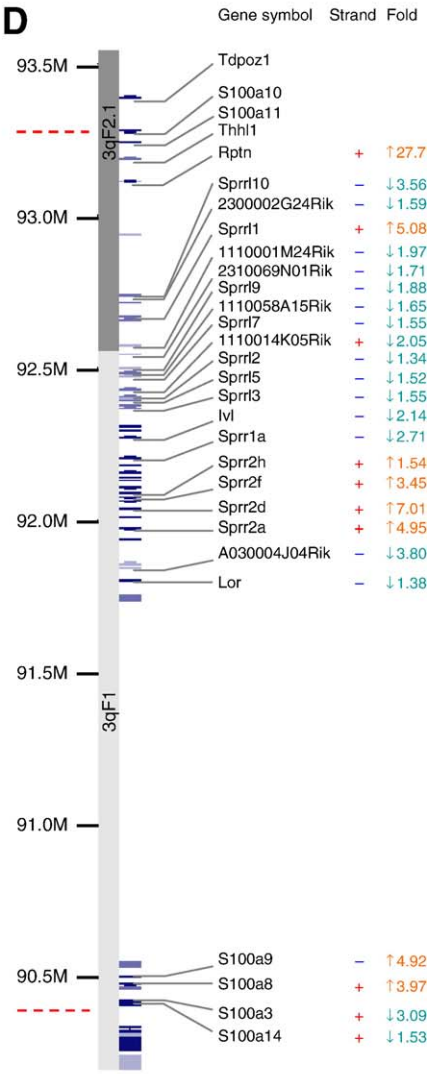
Fig. 6. Deletion of the *Get-1* gene affects the expression of multiple genes crucial for terminal differentiation and barrier function of epidermis. (A) Overview of microarray data processing and Cyber-T analysis to identify statistically significant differentially expressed genes in the backskin of *Get-1*$^{-/-}$ mice. (B) List of differentially expressed genes grouped into functional categories important in epithelial barrier formation. *P* value corresponds to the significance of differential expression as determined by Cyber-T analysis; bold *P* values are highly significant ($P<0.0025$). Asterisk, the probe set corresponding to *Get-1* is outside the deleted exons of *Get-1*$^{-/-}$ mice. (C) Semiquantitative RT-PCR of a random selection of genes from panel B. Asterisk, the primer set used to interrogate the expression of Get-1, is outside the deleted exons of *Get-1*$^{-/-}$ mice. Gapdh and 18S are endogenous controls. (D) Fold changes of gene expression within the epidermal differentiation complex (EDC). For graphical clarity, only genes that are differentially expressed with $P<0.05$ are shown with gene symbol, strand direction and fold change. Dotted lines, boundaries of the EDC.





B

UniGene ID	Gene title	Gene symbol	*P* value	Fold
Structural proteins				
Mm.162684	filaggrin	Flg	0.01959	-1.34
Mm.156955	involucrin	Ivl	**0.00211**	-2.14
Mm.1121	loricrin	Lor	**0.00072**	-1.44
Mm.331191	small proline-rich protein 1A	Sprr1a	**0.00233**	-2.71
Mm.6853	small proline-rich protein 2A	Sprr2a	**<0.00001**	4.95
Mm.87820	small proline-rich protein 2D	Sprr2d	**<0.00001**	7.01
Mm.10692	small proline-rich protein 2F	Sprr2f	0.03228	3.45
Mm.10693	small proline-rich protein 2H	Sprr2h	0.02092	1.54
Mm.301748	small proline rich-like 1	Sprrl1	**<0.00001**	5.08
Mm.279773	small proline rich-like 2	Sprrl2	0.02453	-1.34
Mm.41969	small proline rich-like 3	Sprrl3	0.00436	-1.54
Mm.291769	small proline rich-like 5	Sprrl5	0.00513	-1.52
Mm.176243	small proline rich-like 7	Sprrl7	0.00499	-1.55
Mm.23784	small proline rich-like 9	Sprrl9	0.00444	-1.88
Mm.292457	small proline rich-like 10	Sprrl10	**<0.00001**	-3.56
Mm.1417	repetin	Rptn	**<0.00001**	27.71
Mm.703	S100 calcium binding protein A3	S100a3	0.04121	-3.09
Mm.21567	S100 calcium binding protein A8 (calgranulin A)	S100a8	**0.00009**	3.97
Mm.2128	S100 calcium binding protein A9 (calgranulin B)	S100a9	**<0.00001**	4.92
Mm.24006	S100 calcium binding protein A14	S100a14	0.00492	-1.53
Mm.35806	RIKEN cDNA 2300002G24 gene (homolog to human Nice1)	2300002G24Rik	**0.00187**	-1.59
Adhesion molecules				
Mm.289441	claudin 1	Cldn1	**0.00087**	-2.31
Mm.7339	claudin 4	Cldn4	**0.00058**	-2.20
Mm.37817	claudin 23	Cldn23	**0.00024**	-3.01
Mm.4807	occludin	Ocln	**0.00228**	-2.22
Mm.350037	plakophilin 3	Pkp3	**0.00242**	-1.67
Mm.266875	periplakin	Ppl	**0.00009**	-1.80
Mm.293683	envoplakin	Evpl	0.04208	-1.30
Mm.909	desmocollin 1	Dsc1	0.03673	-1.54
Mm.247477	corneodesmosin	Cdsn	0.01393	-1.49
Protein / lipid-modifying enzymes				
Mm.41964	transglutaminase 1, K polypeptide	Tgm1	**0.00195**	-1.81
Mm.20851	peptidyl arginine deiminase, type III	Padi3	0.00489	-2.28
Protease and serine-protease inhibitor				
Mm.20024	similar to human kallikrein 5 preproprotein	1110030O19Rik	**0.00125**	-1.96
Mm.83909	serine (or cysteine) proteinase inhibitor, clade B, member 8	Serpinb8	0.01608	-1.74
Transcription factors				
-	grainyhead like epithelial transactivator 1 *	Get1 / Grhl3	**0.00112**	-2.00
Mm.325445	Skin-1a	Pou2f3 / Skn-1a	**0.00198**	-1.71
Enzymes and transporters involved in lipid metabolism				
Mm.41989	arachidonate lipoxygenase 3	Aloxe3	0.02175	-1.50
Mm.340329	arachidonate 12-lipoxygenase, 12R type	Alox12b	0.00310	-2.17
Mm.5031	glucosidase, beta, acid	Gba	0.04756	-1.30
Mm.4628	sphingomyelin phosphodiesterase 1, acid lysosomal	Smpd1	**0.00001**	-2.03
Mm.23298	sphingomyelin phosphodiesterase 3, neutral	Smpd3	**0.00049**	-3.14
Mm.287187	sphingomyelin phosphodiesterase, acid-like 3B	Smpdl3b	**0.00196**	-2.54
Mm.249342	7-dehydrocholesterol reductase	Dhcr7	0.00446	-1.66
Mm.5092	phospholipase A2, group IB, pancreas, receptor	Pla2g1br	0.03762	1.50
Mm.331989	phospholipase A2, group IIF	Pla2g2f	0.00301	-2.21
Mm.100476	phospholipase A2, group III	Pla2g3	0.00257	-2.14
Mm.87136	Phospholipase A2, group IVB (cytosolic)	Pla2g4b	**0.00097**	-2.22
Mm.180189	diacylglycerol O-acyltransferase 2	Dgat2	**0.00021**	-1.78
Mm.282096	elongation of very long chain fatty acids-like 1	Elovl1	**0.00012**	-1.65
Mm.281887	glycerophosphodiester phosphodiesterase domain containing 1	Gdpd1	**0.00102**	-2.13
Mm.246881	glycerophosphodiester phosphodiesterase domain containing 3	Gdpd3	**<0.00001**	-2.88



fragile in *Get-1*$^{-/-}$ mice (Fig. 3B), suggesting that the barrier dysfunction is not caused by increased fragility of the cornified envelope. However, we found that the lipid composition of the epidermis was altered with statistically significantly increased phospholipids (PL) and decreased fatty acids (FA), which are implicated in maintaining normal barrier function (Elias, 2005) (Fig. 3C). Because FA are derived from PL, these findings indicate an impairment in the conversion of PL to FA (Fluhr et al., 2001).

To investigate in more detail the epidermal structure of *Get-1*$^{-/-}$ mice, we performed transmission electron microscopy (TEM). These experiments showed that the stratum corneum of the *Get-1*$^{-/-}$ mice was more compact and that the corneocytes were enlarged with irregular surface structure (Figs. 4A–B). The space between the corneocytes, where lipid lamellar layer normally exists, contained residual material not found in wild type (WT) mice (Figs. 4C–D), consistent with abnormalities in lipid composition. Interestingly, intercellular junctions in the top of the granular layer showed abnormal separation of cells between desmosomal regions, creating the appearance of periodic intercellular spaces (Figs. 4E–H). This adhesion abnormality was observed in three out of five knockout mice and not in four WT mice. The lipids synthesized in the cells of the granular layer are packaged into lamellar bodies that are subsequently secreted for use in the intercellular spaces of the stratum corneum. Consistent with abnormal lipid composition, we found that lamellar bodies are decreased in size and optical density in *Get-1*$^{-/-}$ mice (Figs. 4I–L).

Because tight junctions have been shown to be critical for effective epidermal barrier function, we analyzed the epidermal expression of claudin 1, claudin 4 and occludin. As expected, occludin is most highly expressed at the cell surface of keratinocytes in the granular layer of WT mice (Figs. 5A–C). Intriguingly, occludin expression appears displaced from the granular layer but is found aberrantly in the cornified layer of *Get-1*$^{-/-}$ mice (Figs. 5D–F). In contrast, although the pattern of claudin 1 expression appears normal, overall expression appears slightly decreased in the *Get 1*$^{-/-}$ epidermis (Figs. 5G–H). Western blot analysis showed that whereas the skin expression of occludin is increased, claudin 1 protein expression is decreased in the *Get 1*$^{-/-}$ mice (Fig. 5I). In summary, there are abnormalities in the pattern of expression for occludin and altered expression levels for claudin 1. Together, these findings are indicative of multiple causes for the barrier defect of *Get-1*$^{-/-}$ mice, including changes in extracellular lipids and defective cell–cell adhesions in the granular layer.

A

	Probe sets	Unique genes	Promoters retrieved	Binding sites within conserved regions
Downregulated	206	162	137	7
Upregulated	88	69	59	3
Non-regulated	246	192	174	0

B

	Sequence	From	To
Downregulated			
mm6_Anxa9	TGATGGTATGTGTCCC**AACAGGTT**CTTCTCTATCAACCTT	772	779
hg17_Anxa9	TGCTGGTATGTGCCCC**AACAGGTT**TTTCCCTATCAGCCTT	195	202
mm6_Bnipl	GGAGGGGAGGTGGCAA**AACCTGTT**T GAAGATGAGGTTAC	51	58
hg17_Bnipl	GGAGAGGAG TGGCAA**AACCTGTT**TTGAAGATGAGGTTAC	47	54
mm6_2300002G24Rik	CTCTGGTTCAGTAAGC**AACCTGTT**AATCAGGCTCTAAAGC	1135	1142
hg17_c1ORF42 (Nice1)	CTCTAG GAACTGGC**AACCTGTT**AATCAGGCTCTAAATC	1198	1205
mm6_Gsdm1	GAGGCGGGGCACTTCA**AACAGGTT**GATGCTGTTCTCCTGG	123	130
hg17_Gsdm1	GCCAGGGCACACGTGG**AACAGGTT**GATGTTGTTCTCCAGG	81	88
mm6_Gpld1	AAACAACAAAAAGAGA**AGCCGGTT**CTCGGTGATCACCATA	105	112
hg17_Gpld1	AAATACTGAAAAG GA**AGCCAGTT**CCAGCTGATTACCATA	418	425
mm6_Sult2b1	GGGCAGCAGCTGGGAG**AACCGGCT**G TGCCCCTCCC	33	40
hg17_Sult2b1	GGGTAGCAGCTGGGAG**AACCGGCT**GGGTGCTGCCCCTCCC	62	69
mm6_Tgm1	GAGAGTGTGTTCCTGG**AGCCGGTT**GGTCTGGATTCTCAAG	1249	1256
hg17_Tgm1	CAGCACAGGTGCCT G**AGCCGGTT**GGTCTGGATTCCCAGG	854	861
Upregulated			
mm6_Nrk	GAGCGGCGCAGAGCGG**GACCGGTT**TTCCGGGGCTGGCAGC	374	381
hg17_Nrk	GAGCGGCGCGGAGCGA**GACCGGTT**TTCCGGGGCTGGCAGC	320	327
mm6_Rptn	GCATACTACAATGACA**AAACAGGTT**CATGAGCAAACAAGGG	189	196
hg17_Rptn	ACCTACTGTAGTGACA**AAACAGGTT**CATGAGCAAGTGGGGG	178	185
mm6_s100a9	GGGAAGTGAGCCAACT**AACCAGTT**TCCCCCCAAACAAGCT	195	202
hg17_s100a9	GGGAAATGAACTAAAC**AACCAGCT**TCCTCCCAAACCAGTT	225	232

C

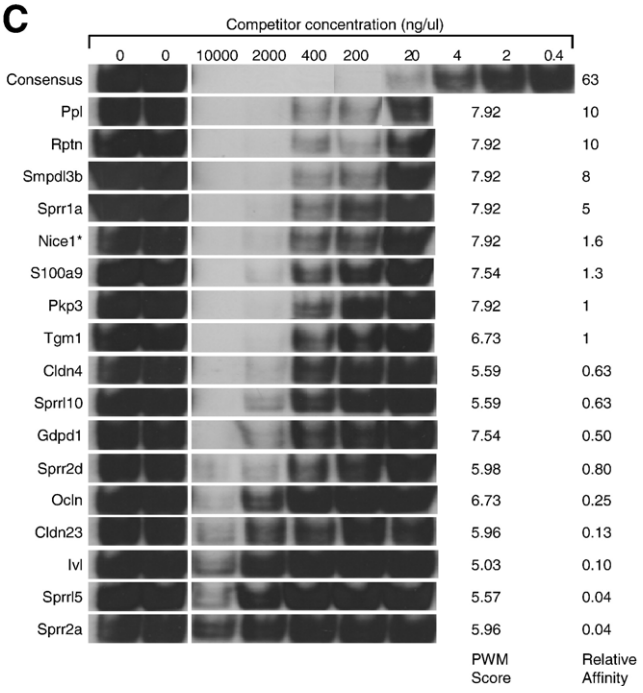


Fig. 7. Identification of evolutionarily conserved Get-1 binding site in promoter regions of *Get-1*-regulated genes. (A) Overview of Get-1 binding site analysis in promoters from differentially expressed genes in *Get-1*$^{-/-}$ epidermis. As a control, we also determined the frequency of Get-1 binding sites in non-regulated genes. (B) Get-1 binding sites in regulated genes. Location of the binding site in relation to the start site is indicated for both human and mouse genes. (C) Electrophoretic mobility shift assays with a ^{32}P-labeled consensus DNA-binding site for the Get-1 protein. Binding was competed with the indicated amounts of unlabeled binding sites from the indicated Get-1-responsive genes. Shown on the right are the probability weight matrix (PWM) scores of potential Get-1 binding sequences as previously determined, and the affinity of each sequence relative to the site found in the Tgm1 gene.

Deletion of the Get-1 gene affects multiple epidermal genes involved in lipid metabolism, cell–cell adhesion and structural integrity of the cornified envelope

To gain insights into the molecular mechanisms of permeability barrier defects in *Get1*$^{-/-}$ mice, we performed expression profiling of backskin RNA from *Get1*$^{-/-}$ and WT mice at e18.5 (Fig. 6A). Using the Cyber-T program (Long et al., 2001) with $P<0.0025$ (false discovery rate <20%), we found that only 294 of the 25,064 expressed probe sets were significantly altered, indicating that deletion of *Get-1* leads to highly selective changes in gene expression. Of the altered probe sets, 206 (162 unique genes) were downregulated, and 88 (69 unique genes) were upregulated (Fig. 6A). A complete list of these genes with corresponding *P*-values is provided in Supplementary Table 4. Strikingly, the great majority of differentially expressed genes play key roles in the terminal differentiation program and barrier formation, including structural proteins of the cornified envelope, cell adhesion molecules and enzymes involved in lipid metabolism (Fig. 6B). A selection of altered genes from different functional groups was validated with semi-quantitative RT-PCR (Fig. 6C).

Systematic statistical analysis of chromosomal location revealed that the significantly downregulated genes were overrepresented in two adjacent chromosomal bands, 3F1 ($P=0.00038$) and 3F2.1 ($P=0.031$). These genes on chromosome 3 fall within the epidermal differentiation complex (EDC), which comprises a large number of genes that encode structural proteins of the cornified envelope (Fig. 6D). Although Get-1 regulates the majority of the genes within the EDC, it is of note that some genes are downregulated whereas others are upregulated. This is in contrast to the Klf4 transcription factor, which represses all *Sprr2* and *Sprr1* genes (Segre et al., 1999), indicating that these two regulatory factors act differently on the EDC. In addition, there is a decrease in the expression of a large number of adhesion molecules, including claudins, occludin, Plakophilin 3 and Periplakin (Fig. 6B) which likely contributes

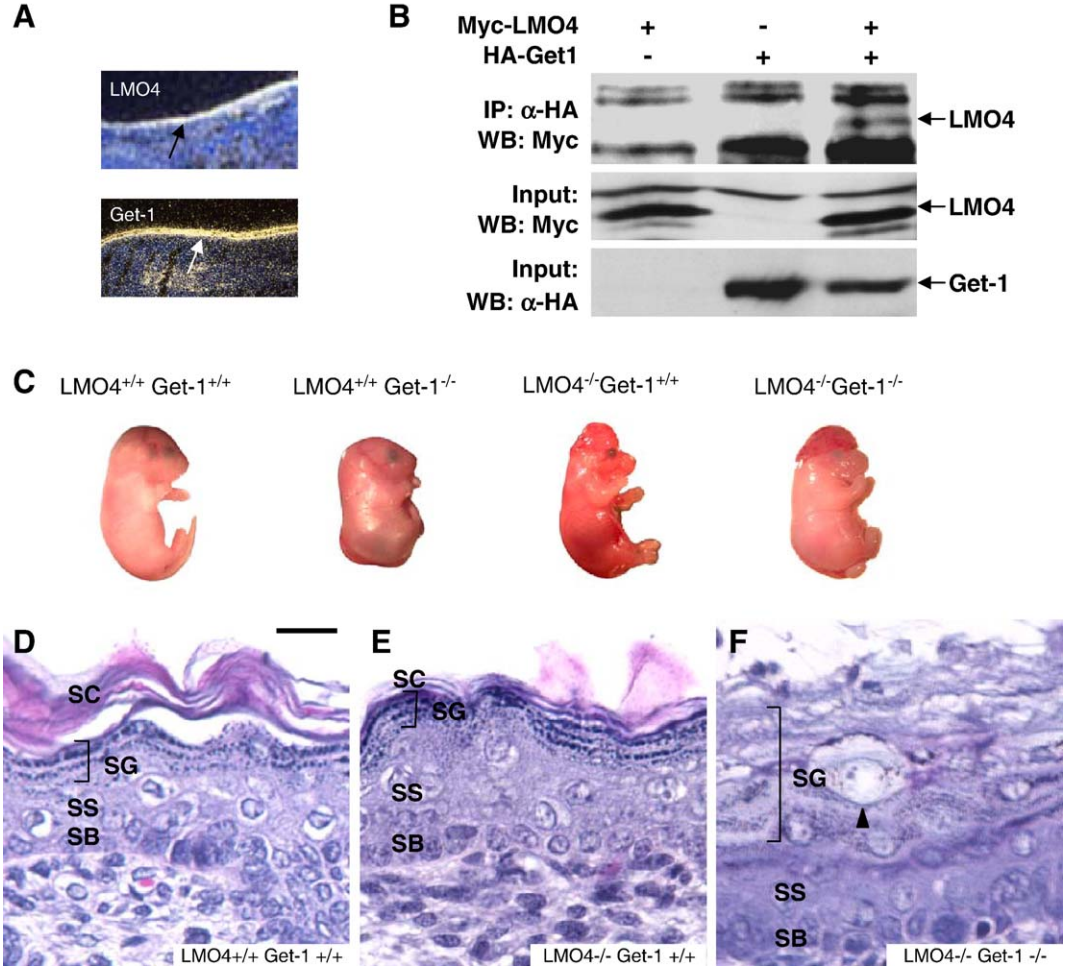


Fig. 8. Get-1 and LMO4 interact functionally to regulate terminal differentiation of the epidermis. (A) *In situ* hybridization showing expression of LMO4 and Get-1 in the developing epidermis at e15. (B) Co-immunoprecipitation of LMO4 and Get-1. Expression vectors encoding Myc tagged (MT) LMO4 and HA-tagged Get-1 were transfected into HEK293T cells either alone or together as indicated on top. The top panel shows a Western blot with MT antibody after immunoprecipitation with an HA antibody. The middle and lower panels show the input detected with MT and HA antibodies, respectively. Prominent bands in the top panel are IgG. The location of LMO4 and Get-1 is indicated with arrows. (C) Phenotypes of WT, *Get-1*$^{-/-}$, *LMO4*$^{-/-}$ and *LMO4*$^{-/-}$*Get-1*$^{-/-}$ e18.5 embryos. (D–F) Histological analysis of e18.5 backskin from WT (D), *LMO4*$^{-/-}$ (E) and *LMO4*$^{-/-}$*Get-1*$^{-/-}$ (F) mice. Scale bar: 25 μm. SB, basal layer; SC, cornified layer; SG, granular layer; SS, spinous layer.

to the adhesion (Figs. 4G–H) and barrier (Fig. 3A) defects of *Get-1*$^{-/-}$ mice.

Extracellular lipids such as free fatty acids, ceramide, cholesterol and cholesterol esters form a lamellar lipid layer between corneocytes and are essential for the barrier function of the epidermis. Several enzymes, including phospholipase A2, acid sphingomyelinase and beta-glucocerebrosidase are involved in the modification of these lipids (Elias, 2005). Among all significantly downregulated genes, we found that the most overrepresented gene ontology biological process category is lipid metabolism ($P=0.000014$), including enzymes and transporters for lipid metabolism (Fig. 6B); nearly all these genes were markedly downregulated. These include several A2 phospholipases that are critical for conversion of PL to FA (Fluhr et al., 2001), which is defective in the *Get-1*$^{-/-}$ mice (Fig. 3C). In addition, several of the downregulated enzymes, including Aloxe3, Alox12b (Jobard et al., 2002) and DGAT2 (Stone et al., 2004), have been shown in gene knockouts to be required for normal barrier function. Taken together, our results suggest that the cause of the defective barrier of *Get-1*$^{-/-}$ mice is multifactorial, involving structural components, cell–cell adhesion and lipid metabolism.

To discover potential Get-1 binding sites in differentially expressed genes, we extracted 2 kb sequences upstream of the

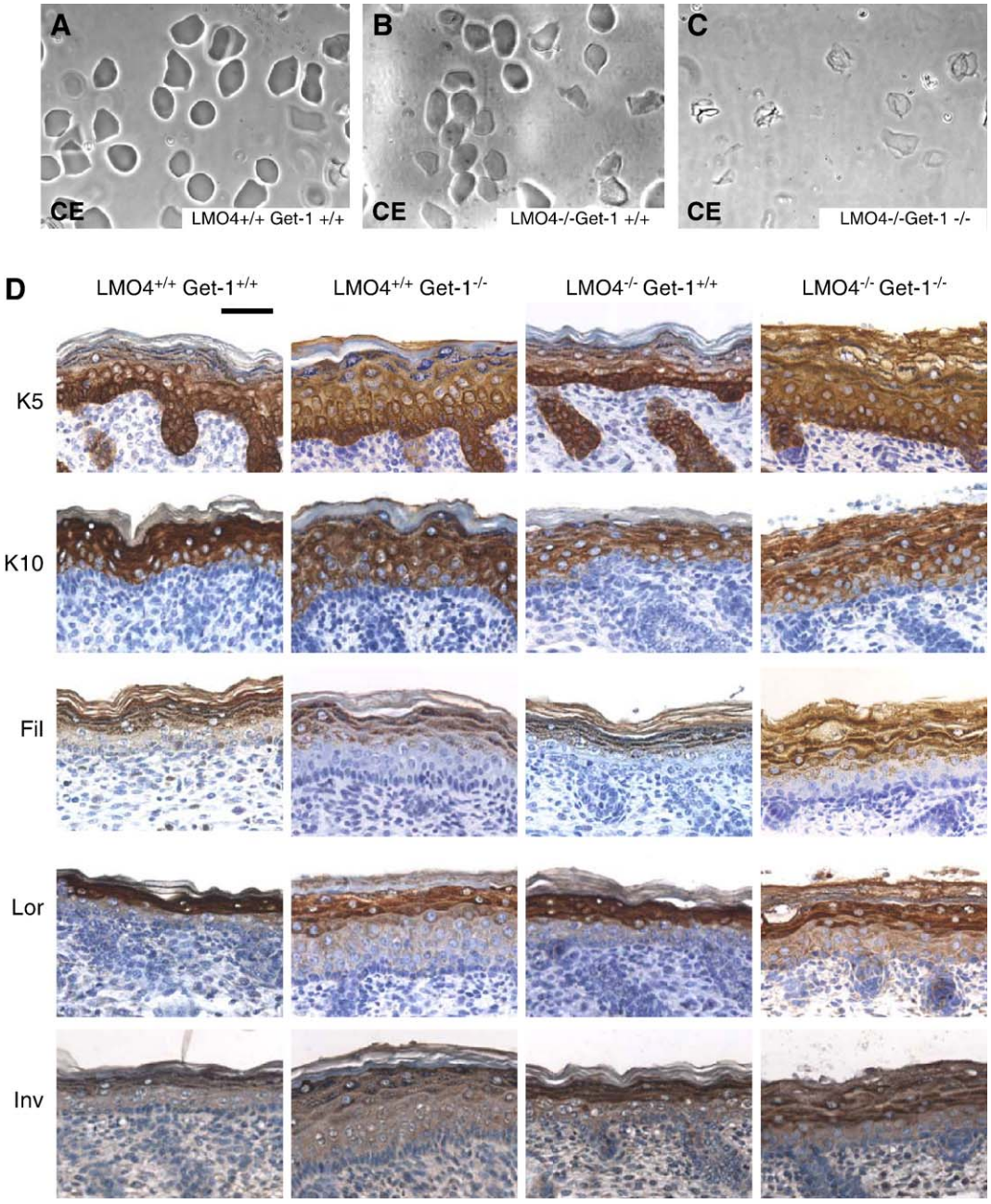


Fig. 9. Impaired stratum corneum formation in *LMO4*$^{-/-}$*Get-1*$^{-/-}$ epidermis. (A–C) Cornified envelopes isolated from e18.5 epidermis from WT (A), *LMO4*$^{-/-}$ (B) and *LMO4*$^{-/-}$*Get-1*$^{-/-}$ epidermis (C). (D) Immunostaining analysis of e18.5 epidermis from embryos of the indicated genotypes. The antibodies used are shown on the left hand. Scale bar: 50 μm. CE, cornified envelope.

transcription start site of these genes and performed positional weight matrix (PWM) analysis (Fig. 7A). Because *Tgm1* is the only known putative target gene of Get-1, we used the PWM score of the potential Get-1 binding site in the upstream region of Tgm1 (6.73) as the cut-off. Using the ConSite program (Sandelin et al., 2004), we determined the mouse–human conservation of the potential binding site as well as the surrounding genomic region. We thus found seven down-regulated genes and three upregulated genes with highly conserved sites, most of them linked to terminal differentiation of keratinocytes (Eckert et al., 2004; Higashi et al., 2004; Koch et al., 2000; Marenholz et al., 2001; Xie et al., 1993). No conserved sites were found in the non-regulated genes, indicating that the presence of high affinity Get-1 binding sites in these genes cannot be explained by chance alone (Fig. 7A). The conserved sites are all found within highly conserved genomic regions suggesting that they are part of gene-regulatory modules (Fig. 7B). Whereas this analysis suggests that some of the differentially expressed genes in the *Get-1*$^{-/-}$ epidermis could be direct target genes, further experiments with *in vivo* Get-1 binding and mutagenesis in transgenic mice are required for identification of direct target genes. We tested a random selection of potential Get-1 binding sites, some of which are in the conserved regions, and found a good correlation between binding affinity of the sites and their PWM score, thus validating previously described consensus sequence (Ting et al., 2005) (Fig. 7C). Interestingly, both up- and down-regulated genes contain conserved high-affinity binding sites, suggesting that Get-1 can act as an activator and repressor on distinct target genes (Kudryavtseva et al., 2003).

Get-1 interacts functionally with LMO4 to regulate terminal differentiation of the epidermis

We reported that Get-1 and LMO4 interact *in vitro* and have overlapping expression patterns during epidermal development (Fig. 8A) (Kudryavtseva et al., 2003). Consistent with previous results of GST pulldown assays and yeast two-hybrid interactions assays, Get-1 and LMO4 co-immunoprecipitate when transfected into HEK293T cells (Fig. 8B). However, the epidermal phenotype of *LMO4*$^{-/-}$ mice has not been characterized, and it is not known whether there are genetic interactions between *LMO4* and *Get-1* during development, including epidermal differentiation. Similar to *Get-1*$^{-/-}$ mice, *LMO4*$^{-/-}$ mice die at birth and exhibit abnormal neural tube closure, although more frequently at the anterior end (Lee et al., 2005) (Fig. 8C). In contrast to *Get-1*$^{-/-}$ mice, the epidermal barrier forms normally in *LMO4*$^{-/-}$ mice (Supplementary Fig. 2). To test the hypothesis that there are genetic interactions between *Get-1* and *LMO4*, we crossbred *LMO4*$^{+/-}$ and *Get-1*$^{+/-}$ mice to generate double knockout mice. *Get1/LMO4* double knockout mice show significantly more frequent exencephaly (100%) than that found in single knockouts (14% for *Get-1*$^{-/-}$ and 55% for *LMO4*$^{-/-}$). Similarly, open-eye phenotype was more penetrant in double knockout mice (*Get-1*$^{-/-}$, 7%; *LMO4*$^{-/-}$, 16%; and *Get-1*$^{-/-}$*LMO4*$^{-/-}$, 54%), consistent with a genetic interaction between these two genes (Fig. 8C and Supplementary Table 3). To study epidermal differentiation, we isolated backskin from e18.5 mice representing the four genotypes: WT, *Get1*$^{-/-}$, *LMO4*$^{-/-}$ and *Get1*$^{-/-}$*LMO4*$^{-/-}$. Whereas LMO4 knockout skin did not exhibit clear morphological abnormalities, compared to *Get-1*$^{-/-}$ mice, skin from double knockout mice showed marked impairment of stratum corneum formation with most cells in the top of the epidermis containing nuclei (Figs. 8D–F). Many cells showed enlarged vacuolar-like structure not normally found in the granular layer of the epidermis. This abnormality was primarily found at the anterior part of the embryo in the skin covering the head and upper body regions.

To further demonstrate the failure of epidermal terminal differentiation, we isolated cornified envelopes from WT, *LMO4*$^{-/-}$ and *Get1*$^{-/-}$*LMO4*$^{-/-}$ mice and found that although *LMO4*$^{-/-}$ cornified envelopes are normal, they were essentially absent in the double knockout mice (Figs. 9A–C). These findings indicate a severe abnormality in cornified layer formation, consistent with the expression of epidermal protein markers K5, K10, filaggrin, loricrin and involucrin, which are not normally detected in the cornified layer (Fig. 9D). The enhancement of the terminal differentiation defect in the double knockouts indicates that *Get-1* and *LMO4* interact functionally to regulate this process.

Discussion

Expression of the *Get-1* gene is initiated in the somatic ectoderm prior to formation of epidermis and continues in the proliferating cells of the developing epidermis (Fig. 8A). By birth, however, the expression level has decreased and transcripts are limited to the suprabasal compartment of the epidermis (Kudryavtseva et al., 2003). In light of the early expression of Get-1, it is interesting that the gene is critical for the terminal differentiation of keratinocytes later in embryogenesis. These results suggest that the terminal differentiation program of epidermis is initiated relatively early during skin development. In addition to epidermis, the *Get-1* gene is expressed in endoderm-derived epithelia such as that of the gut, the respiratory track and the genitourinary system (Kudryavtseva et al., 2003). Consistent with these data, we now show that the function of *Get-1* is not limited to ectodermal epithelia. The forestomach epithelium, which is highly similar to the epidermis, exhibits impaired terminal differentiation with a striking decrease in the expression of the terminal differentiation markers loricrin and filaggrin (Figs. 2S–V).

Increased epidermal thickness with expansion of all epidermal layers, especially the granular layer, is a prominent feature of the *Get-1*$^{-/-}$ phenotype. Increased proliferation of basal cell layer keratinocytes (Figs. 2P and Q) accounts for part of the epidermal thickening, but impaired cell death in the top of the granular layer may also contribute to this abnormality. Thus, we noticed frequent persistence of nuclei in the stratum corneum (Fig. 2C) and decreased caspase 14 expression in the granular layer of *Get-1*$^{-/-}$ mice (data not shown). The increased epidermal proliferation is not likely a cell-autonomous effect of Get-1 because proliferation is upregulated in the basal cell layer

at a stage when Get-1 expression is limited to the suprabasal compartment.

In addition to hyperplasia, the main epidermal abnormalities in *Get-1*$^{-/-}$ mice are found in the cornified and granular layers. Keratinocytes in the granular layer show defective flattening (Figs. 2B–C) and abnormal cell–cell adhesion (Figs. 4E–H). The cornified layer shows striking abnormalities with increased thickness, tighter cell–cell adhesion and frequent persistence of nuclei (Figs. 2C and 3B). The persistence of nuclei in the stratum corneum indicates impairment of enucleation at the top of the granular layer, an important component of the terminal differentiation program. From a functional standpoint these morphological abnormalities are significant because the barrier function of *Get-1*$^{-/-}$ mice is severely impaired (Fig. 3A).

We performed gene expression profiling to gain insights into the molecular mechanisms underlying the morphological and functional defects in the *Get-1*$^{-/-}$ epidermis (Fig. 6). The results suggest that alterations in the expression of multiple genes encoding key functional components of the terminal differentiation program contribute to defective epidermal barrier. In particular, our findings indicate that lipid abnormalities may cause defective barrier function of *Get-1*$^{-/-}$ mice. We found increased PL and decreased FA (Fig. 3C), which is implicated in maintaining normal barrier function (Elias, 2005). This finding is consistent with decreased expression of several A2 phospholipases (Fluhr et al., 2001) (Fig. 6B) that are critical for conversion of PL to FA (Fluhr et al., 2001). Altered appearance of lamellar bodies (Figs. 4I–L), as well as defective lipid lamellar formation in the cornified layer (Figs. 4C–D), also support an important role for Get-1 in regulation of epidermal lipids.

In addition to regulating lipids we found that Get-1 plays an important role in regulating transcripts involved in cell–cell adhesion, a key feature of an effective epidermal barrier. In the granular layer, there are three different cell junctions–desmosomes, tight junctions and adherence junctions–responsible for "sealing" the membranes of adjacent cells and maintaining barrier function. Knockouts of tight junction proteins (Furuse et al., 2002) and desmosomal proteins (Chidgey et al., 2001) lead to barrier defects. Therefore, the decrease in the expression of a large number of tight junction molecules, including several claudins and occludin (Figs. 6B–C), may contribute to the adhesion (Figs. 4G–H) and barrier (Fig. 3A) defects of *Get-1*$^{-/-}$ mice. Most strikingly, we found that the distribution of occludin is altered with decreased expression in the granular layer and aberrant expression in the cornified layer of *Get-1*$^{-/-}$ mice (Figs. 5D–F). Desmosomal defects could also be involved because a similar cell–cell adhesion abnormality was found in the granular layer of mice ectopically expressing Desmoglein 3 in the suprabasal compartment (Merritt et al., 2002). Consistent with this notion, Plakophilin 3 and Periplakin (Figs. 6B–C) were significantly downregulated in *Get-1*$^{-/-}$ epidermis.

Get-1 is also critical for normal expression of many structural components of the cornified envelope such as involucrin, Sprr1a, Sprrl10 and S100a3. Yet, the strength of the cornified envelope of *Get-1*$^{-/-}$ mice appeared normal (Fig. 3B). One possible explanation for this apparent dichotomy is that several structural components, including Sprr2d, Sprr2h and Repetin, were upregulated, thus compensating for the decreased expression of other components as has been suggested in loricrin knockout mice (Koch et al., 2000).

Taken together, our results strongly suggest that Get-1 plays a broad role in terminal differentiation of the epidermis and that the defective epidermal barrier of *Get-1*$^{-/-}$ mice is due to alterations in the expression of multiple genes. Consistent with this idea, the epidermal phenotype of *Get-1*$^{-/-}$ mice is striking whereas the magnitude of change for most genes in *Get-1*$^{-/-}$ epidermis is quite modest. The abnormal permeability barrier of *Get-1*$^{-/-}$ mice was previously proposed to result from decreased Tgm1 expression without the involvement of other factors involved in barrier formation (Ting et al., 2005). Our findings directly contradict these conclusions, and for several reasons we think that downregulation of Tgm1 is unlikely to fully explain the epidermal phenotype of *Get-1*$^{-/-}$ mice. First, although the barrier defect is striking, the downregulation of Tgm1 is modest, approximately two-fold. Second, although *Tgm1*$^{-/-}$ mice exhibit dramatic defects in CE formation and strength (Kuramoto et al., 2002), the fragility of the CE is decreased if anything in *Get-1*$^{-/-}$ mice (Fig. 3B), perhaps due to functional overcompensation of cornified envelope proteins such as Repetin, and certain S100 and Sprr proteins. Third, we have demonstrated altered expression of transcripts for many other key molecules involved in terminal differentiation of the epidermis and barrier formation.

To begin understanding the transcriptional mechanisms underlying gene expression changes in *Get-1*$^{-/-}$ epidermis, we have studied the frequency of Get-1 binding sites in the regulatory regions of differentially expressed genes (Fig. 7A). Although the data does not allow us to determine which of these genes are direct targets of Get-1, we have obtained clear statistical evidence that Get-1 binding sites are enriched in Get-1-responsive genes. Interestingly, however, Get-1-binding sites are absent in the conserved regions of the 2-kb of 5′ flanking sequence for most genes altered in *Get-1*$^{-/-}$ epidermis. These genes may well contain Get-1 binding sites at a greater distance from the start site. For example, a locus control region at a distance may regulate the EDC locus (Martin et al., 2004), which contains many Get-1-responsive genes (Fig. 6D). Heretofore, Klf4 is the only transcription factor conclusively shown to regulate multiple genes of the EDC (Patel et al., 2003; Segre et al., 1999). However, Get-1 appears to act differently than Klf4 because in the *Get-1*$^{-/-}$ mice some of the EDC genes are upregulated while others are downregulated. Some transcripts may also be altered in response to the barrier defect, injury and epidermal hyperplasia of *Get-1*$^{-/-}$ mice. These include Sprr2 family members, also upregulated in the loricrin knockout mice (Koch et al., 2000), as well as S100A8 and S100A9, which are downstream of AP1 (Figs. 6B and C) (Zenz et al., 2005). Interestingly, both S100A9 and Repetin transcript levels were increased at least as early as e16.5 (Supplementary Fig. 3). Based on the expression analysis, it is unlikely that alteration in the expression of other transcription factors mediate all actions of Get-1. Expression of Klf4 is not significantly decreased in the

Get-1$^{-/-}$ skin, and the modest decrease in the expression of Skn-1, a POU domain factor involved in epidermal differentiation, is unlikely to be a major contributor because singular deletion of the *Skn-1* gene causes a very mild abnormality in the terminal differentiation of the epidermis (Andersen et al., 1997).

Because we cloned Get-1 as an LMO4-interacting protein (Kudryavtseva et al., 2003), it was of interest to determine whether there are interactions between the two genes in epidermal development. Gene knockouts for both *LMO4* (Hahm et al., 2004; Lee et al., 2005; Tse et al., 2004) and *Get-1* (Ting et al., 2003a) result in neural tube defects, which support the possibility that interaction between the two proteins is important. Interestingly, however, the epidermis of *LMO4*$^{-/-}$ mice appears normal (Figs. 8D–E; Supplementary Fig. 2), indicating that under non-perturbed conditions, Get-1 can function without LMO4. Yet, deletion of the *Get-1* gene in the absence of the *LMO4* gene leads to a much more striking phenotype with a dramatic impairment in the formation of the cornified layer, and a markedly abnormal granular layer (Figs. 8F and 9). Furthermore, the penetrance of the eye-open and exencephaly phenotypes is significantly increased in the double knockouts compared to either single knockout (Supplementary Table 3). Together, these findings demonstrate genetic interactions between *LMO4* and *Get-1* and based on the ability of these proteins to interact *in vitro*, it is tempting to speculate that the functional interaction results from altered transcriptional complexes in which both LMO4 and Get-1 participate. It is also possible that LMO4 and Get-1 play sequential roles at different stages in the control of epidermal development.

In conclusion, our results suggest that *Get-1* plays an important role in the terminal differentiation and barrier function of the epidermis by regulating multiple genes, including those of the EDC complex encoding structural proteins, enzymes controlling extracellular lipid metabolism, cell adhesion molecules and protein-modifying enzymes. Furthermore, *Get-1* interacts functionally with *LMO4* to regulate terminal differentiation of the epidermis. The broad range of genes whose expression is altered by *Get-1* deletion implicates Get-1 proximally in a complex pathway that ultimately coordinates different aspects of the terminal differentiation program in epidermis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.07.015.

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Session Title: Repression of Gene Expression

#1613 The LIM-only factor LMO4 regulates expression of the BMP7 gene through an HDAC2-dependent mechanism, and controls cell proliferation and apoptosis of mammary epithelial cells. Zhongxian Lu[1], Ning Wang[1], Kevin Lin[1], Kaye Starr Lam[1], Xiaoman Xu[1], Zhengquan Yu[1], Gordon N. Gill[2], Bogi Andersen[1]. [1]University of California, Irvine, Irvine, CA and [2]Department of Medicine, University of California, San Diego, La Jolla, CA.

The nuclear LIM-only protein LMO4 is upregulated in breast cancer, especially estrogen receptor negative tumors, and its overexpression in mice leads to hyperplasia and tumor formation. Here, we show that deletion of *LMO4* in the mammary glands of mice leads to impaired lobuloalveolar development due to decreased epithelial cell proliferation. With the goal of discovering potential LMO4-target genes, we also developed a conditional expression system in MCF-7 cells for both LMO4 and a dominant negative (DN) form of its co-regulator, Co-factor of LIM domains (Clim/Ldb/Nli). We then used DNA microarrays to identify genes responsive to LMO4 and DN-Clim upregulation. One of the genes common to both datasets was *BMP7*, whose expression is also significantly correlated with LMO4 transcript levels in a large dataset of human breast cancers, suggesting that *BMP7* is a *bona fide* target gene of LMO4 in breast cancer. Inhibition of BMP7 partially blocks the effects of LMO4 on apoptosis, indicating that BMP7 mediates at least some of functions of LMO4. Gene transfer studies show that LMO4 regulates the BMP7 promoter, and chromatin immunoprecipitation studies show that LMO4 and its co-factor Clim2 are recruited to the BMP7 promoter. Furthermore, we demonstrate that HDAC2 recruitment to the BMP7 promoter is inhibited by upregulation of LMO4 and that HDAC2 knockdown upregulates the promoter. These studies suggest a novel mechanism of action for LMO4: LMO4, Clim2 and HDAC2 are part of a transcriptional complex, and increased LMO4 levels can disrupt the complex, leading to decreased HDAC2 recruitment and increased promoter activity.

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ABSTRACT FORM

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TGFβ signals have multiple and complex biological effects in mammary gland development and tumor progression. By modulating the binding and activity of Smad proteins on target genes, Smad-associating proteins are thought to play key roles in TGFβ signal transduction by affecting the specificity and magnitude of TGFβ signal in response to environmental effects. Here, we identified a new Smad-associating protein, LIM-only protein 4 (LMO4), which plays critical roles in mammalian development, and has been proposed to play roles in epithelial oncogenesis, including breast cancer. Using co-immunoprecipitation, we found the interaction between endogenous and transiently overexpressed LMO4, and receptor-mediated Smad proteins (R-Smads). GST pull-down assays confirmed this interaction, and showed that LMO4 can interact with the MH1 and linker domains of R-Smad proteins. Furthermore, LMO4 associated with the endogenous TGFβ-responsive Plasminogen Activator Inhibitor-1 gene promoter in a TGFβ-dependent manner, suggesting that LMO4 may modulate TGFβ signaling as a Smad-associating protein in a biologically relevant manner. To test this hypothesis, we assessed the effect of LMO4 on TGFβ signaling, using a TGFβ-responsive reporter gene. The results show that the transcriptional response to TGFβ in epithelial cells is sensitive to LMO4 levels; both up- and down-regulation of LMO4 can enhance TGFβ signaling. When introduced into mammary epithelial cells, LMO4 leads to enhanced stimulation of p21 and p27, as well as potentiation of the growth-inhibitory effects of TGFβ in those cells. These results define a new function for LMO4 as a coactivator in TGFβ signaling, and provide a novel mechanism for LMO4-mediated regulation in development and oncogenesis. Because LMO4 is expressed at highly different levels during mammary gland development and tumor progression, our results provide insights into how TGFβ signaling may be modified in response to specific environmental effects both in development and during cancer progression in the mammary gland.

Abstract Title:	**A Novel Smad-associating Protein, LIM-only protein 4 (LMO4), Modulates TGFβ Signaling in Mammary Gland Epithelial Cells**
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THE LIM-ONLY PROTEIN LMO4 MODULATES TGFB SIGNALING BY INTERACTING WITH SMAD PROTEINS

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LIM-only protein 4 (LMO4) has been established to have crucial functions in development. In addition, it is often overexpressed in breast cancer cases and is proposed to play roles in oncogenesis. LMO4 is highly expressed in locations of epithelial-mesenchymal interactions where TGFβ cytokine signaling plays important roles, both in development and in cancer. We therefore explored the possibility that LMO4 might modulate TGFβ signaling in breast cancer cells. Primary human mammary gland epithelial (HME) cells were infected with retroviruses expressing LMO4 or control proteins. We found that LMO4 potentiated the TGFβ-mediated inhibition of cell proliferation in HME cells, indicating that under these conditions LMO4 has positive effects on TGFβ function. Consistent with this finding, in transient transfection assays, LMO4 potentiated the TGFβ effect on the well known TGFβ target gene, PAI-1 in 293T, HMEC, and mouse mammary gland epithelial cells (NMuMg). To test the mechanisms of action for LMO4, we co-transfected myc-tagged LMO4 and HA-tagged Smads proteins 1, -2, -4, -5 into 293T cells, and found that LMO4 interacts with these Smads. GST protein-protein interaction assays showed that LMO4 binds to the MH1 and linker domain of Smad1, Smad3 and Smad4. Together, these experiments suggest that LMO4 modulates TGFβ signaling by binding directly to Smad proteins. In support of this idea, we found in chromatin immunoprecipitation assays that after TGFβ treatment LMO4 is part of the Smad-containing transcriptional protein complex on the PAI-1 gene. In summary, we have found that the transcriptional adapter LMO4 may regulate the binding of transcription factors on TGFβ target genes. These results define a new function for LMO4 as a coactivator of TGFβ signaling, and provide a novel mechanism for LMO4 regulation in development and oncogenesis.

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4485 The LIM-only protein LMO4 modulates TGFβ signaling by interacting with Smad proteins

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LIM-only protein 4 (LMO4) has been established to have crucial functions in development. In addition, it is often overexpressed in breast cancer and is proposed to play roles in oncogenesis. LMO4 is highly expressed in locations of epithelial-mesenchymal interactions where TGFβ cytokine signaling plays important roles, both in development and in cancer. We therefore explored the possibility that LMO4 might modulate TGFβ signaling in breast cancer cells. Primary human mammary gland epithelial (HME) cells were infected with retroviruses expressing LMO4 or control proteins. We found that LMO4 potentiated TGFβ-mediated inhibition of cell proliferation in HME cells, indicating that under these conditions LMO4 has positive effects on TGFβ function. Consistent with this finding, in transient transfection assays, LMO4 potentiated the TGFβ effect on the well known TGFβ target gene, PAI-1. To test the mechanisms of action for LMO4, we co-transfected myc-tagged LMO4 and HA-tagged Smad 1, -2, -4 and -5 into 293T cells, and found that LMO4 interacts with these Smad proteins. In support of this idea, we found in chromatin immunoprecipitation assays that after TGFβ treatment LMO4 was part of the Smad-containing transcriptional protein complex on the PAI-1 gene. In summary, we have found that the transcriptional adapter LMO4 may regulate the activity of transcription factors on TGFβ target genes. These results define a new function for LMO4 as a co-factor in TGFβ signaling, and provide a novel mechanism for LMO4 regulation in development and oncogenesis. Acknowledgements: This work was supported by the U.S. Army Medical Research and Materiel Command W81XWH0410483 (to Z.L.) and the NIH AR44882 (to B.A.).

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